TRYPANOSOMA (NANNOMONAS) CONGOLENSE: PATHOGENESIS AND CELLULAR RESPONSES DURING THE EARLY STAGES OF INFECTION IN SHEEP

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DEDICATION

To my wife, Mary Wangari and our son Mwangi for their
love, support and sacrifice and for their courage
and perseverance despite the loneliness they
endured during my absence

DECLARATION

I hereby declare that the work presented in this thesis was carried out entirely by myself except where specifically stated in the text and in the acknowledgements section

Duncan M. Mwangi

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ABBREVIATIONS

ADCC	-	Antibody dependent cell mediated cytotoxicity
APC	-	Antigen presenting cell
BSA	-	Bovine serum albumin
°C	-	Degrees centrigrade
CD	-	Cluster of differentiation
CM	-	Centimetre(s)
CTVM	-	Centre for Tropical Veterinary Medicine
DAB	-	Diaminobenzidine tetrahydrochloride
DAS/Ig	-	Donkey anti-sheep immunoglobulin
DE-52	-	Diethylaminoethyl cellulose
DNA	-	Deoxyribonucleic acid
DNFB	-	Dinitrofluorobenzene
DTH	-	Delayed type hypersensitivity
EDTA	-	Ethylenediaminetetracetate
$F'ab_2$	-	Fraction antigen binding portion of the immunoglobulin molecule
FcR	-	Receptors on cells which bind the crystallizable fraction of immunoglobulin molecule
FITC	-	Fluorescein isothiocyanate
FSC	-	Forward light scatter
$\tau\delta$	-	Gamma/delta
GAM/Ig	-	Goat anti-mouse immunoglobulin
HBSS	-	Hanks balanced salt solution
Hr	-	Hour
HRPO	-	Horseradish peroxidase
Ig	-	Immunoglobulin
IgG	-	Immunoglobulin G
IFN-y	-	Interferon-gamma
IL-2	-	Interleukin-2
IL-2R	-	Interleukin-2 receptor(s)
IMFB	-	Immunofluorescence buffer
IMPB	-	Immunoperoxidase buffer
IPP	-	Ileal Peyer's patches
IU	-	International units
KD	-	Kilodalton
l	-	Litre(s)
LCA	-	Leucocyte common antigen
MAb	-	Monoclonal antibody
MHC	-	Major histocompatibility complex
ml	-	Millilitre(s)
Min.	-	Minutes
mm	-	Millimetre(s)
MNC	-	Mononuclear cells
MV	-	Millivolts
M-VATs	-	Metacyclic variable antigen types
MW	-	Molecular weight
μ	-	Microns
μg	-	Micrograms
μl	-	Microlitre(s)
μm	-	Micrometre(s)
NMS	-	Normal mouse serum
NSS	-	Normal sheep serum
OLA	-	Ovine leucocyte antigens

PAGE	-	Polyacarylamide gel electrophoresis
PAO	-	Polyamine oxidase
PBL	-	Peripheral blood leucocytes
PBS	-	Phosphate buffered saline
PCV	-	Packed cell volume
%	-	Percent
PGE_2	-	Prostaglandin E_2
PMT	-	Photomultiplier tubes
PSG	-	Phosphate saline glucose
RAM/Ig	-	Rabbit anti-mouse immunoglobulins
RPE	-	R-phycoerythrin
SBU	-	Sheep Biology Unit
SDS	-	Sodium dodecyl sulphate
SIg	-	Surface immunoglobulin
SSC	-	Side light scatter
TEM	-	Transmission electron microscopy
T_H	-	T helper cell
TNF	-	Tumor necrosis factor
TREU	-	Trypanosome Research Edinburgh University
TWBC	-	Total white blood cells
UV^-	-	Ultraviolet light
VAT	-	Variable antigen types
VPM	-	Veterinary Pathology, Moredun
VSG	-	Variant surface glycoprotein

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Cellular responses and parasites kinetics associated with the development of local skin reactions were examined in sheep following infection with metacyclic forms of *Trypanosoma (Nannomonas) congolense*. Leucocyte phenotypes in the skin and regional draining lymph nodes were examined by indirect immunoperoxidase staining using monoclonal antibodies specific for ovine leucocyte subsets. Immunofluorescence and flow cytometry was used to determine changes in the numbers and proportions of different cell phenotypes in peripheral blood and in lymph from cannulated afferent and efferent lymphatic ducts draining both the skin reaction and regional nodes respectively. Cellular reactions occurring in the skin prior to development of local skin reactions were investigated by light and transmission electron microscopy.

Trypanosomes were observed in the skin during the first four days of infection. Following development of the local skin reaction, and histological demonstration of trypanosomes in the skin and draining lymph nodes five to seven days after infection, large numbers of parasites appeared in the afferent lymph, reaching peak numbers seven to 10 days after infection. During this period, trypanosomes were also present in the efferent lymph. Trypanosomes were not detectable in skin or draining lymph nodes after 13 days but persisted at a low level in both afferent and efferent lymph. Parasites did not appear in peripheral blood until 15 days after infection.

Cellular responses varied according to the stage of development of local skin reactions and the presence of trypanosomes in the various compartments. Prior to development of local skin reactions, the only cellular event observed histologically by transmission electron microscopy was evidence of mast cell degranulation. Local skin reactions developed at inoculation sites of sheep from day five after infection and were initially characterized by an infiltrate of mononuclear cells and neutrophils. Mononuclear cell infiltrate comprised equal proportions of T cells ($CD5^+$, $CD4^+$ and $CD8^+$) and B cells ($CD45R^+$), MHC Class II^+ cells and macrophages. B cells and MHC Class II^+ cells were found mainly in aggregates, suggesting local proliferation and antibody production. There was a greater proportion of $CD4^+$ cells than $CD8^+$ cells, but SBU-$T19^+$ ($\tau\delta$ T cells) were rarely present. The marked cellular response in afferent lymph during this time was characterized by both an absolute and proportional increase in T cells, particularly those expressing CD4. Expansion of the B cell population was observed in draining lymph nodes while a predominantly lymphoblastic, B cell ($CD45R^+$, SIg^+) and MHC Class II^+ cell response was observed in efferent lymph.

As the local skin reaction started to regress 10 days after infection, the cell infiltrate was predominantly mononuclear, composed of equal numbers of $CD4^+$ and $CD8^+$ cells and a few B cells, MHC Class II^+ cells and SBU-$T19^+$ cells. The afferent lymph still contained high numbers but lower proportions of T cells ($CD4^+$ and $CD8^+$ cells) and also increased numbers and proportions of lymphoblasts, B cells ($CD45R^+$, SIg^+ cells) and MHC Class II^+ cells indicating that these cells were leaving the skin. The B cell response persisted in draining regional nodes and efferent lymph. Changes in peripheral blood leucocyte subpopulations did not occur until 15 to 38 days after infection when a gradual increase in B cells and MHC Class II^+ cells and decline in T cells was observed.

Observations of afferent lymph using light and transmission electron miscroscopy showed that trypanosomes were attached to, and phagocytosed by macrophages/dendritic cells. Further evidence of the host-effector response was the presence of numerous lysed trypanosomes in the skin, afferent and efferent lymph from day eight, suggesting that lytic antibodies were being produced.

Sheep immunized by infection and trypanocidal drug therapy failed to develop local skin reactions following challenge with an homologous serodeme: trypanosomes were absent from the afferent lymph and there was no evidence of a cellular response. Similarly, following challenge of infected sheep with an heterologous serodeme, skin reactions failed to develop, no trypanosomes were seen in draining lymphatics and there was no evidence of a cellular response in efferent lymph. Hence, both homologous immunity and interference in establishment of secondary infection appears to operate at the level of the skin.

Proliferation of trypanosomes in the skin is crucial for establishment of infection. The marked cellular responses elicited in the skin and draining lymph nodes are however ineffective in preventing the onset of infection but are important in subsequent development of homologous immunity following infection and therapy.

CHAPTER ONE
GENERAL INTRODUCTION

The African trypanosomiases are a complex of diseases of man and his domesticated livestock caused by several different species of haemoprotozoan parasites of the genus *Trypanosoma.* Trypanosomes are transmitted by haematophagous flies of the genus *Glossina* (Mulligan, 1970). The parasites are ingested together with a bloodmeal when the flies feed on an infected host. In the insect vector, trypanosomes undergo a cycle of development in the alimentary tract eventually maturing into infective metacyclic trypanosomes. The distribution of African trypanosomiases follows closely that of the vector. Tsetse flies infest an area of some 10 million km^2, nearly 37% of the African continent south of the Sahara (Finelle, 1980) and affect a total of 37 countries (FAO/WHO/OIE, 1982). Two thirds of this land mass is potentially utilizable for both livestock and crop production (World Animal Review, 1983). Trypanosomiases in Africa poses a threat to more than 45 million people (UNDP/World Bank/WHO, 1983), 147 million cattle (FAO/WHO/OIE, 1982), 125 million goats and 103 million sheep (Dwinger, 1985).

Trypanosomes causing disease in domesticated livestock were first discovered in the late 19th century. Evans (1880), identified a trypanosome later named *Trypanosoma evansi* in camels and Indian horses suffering from the disease known as 'surra' and established the link between the disease and its aetiological agent. Bruce (1895) reported the occurrence in South Africa of a parasite identical to or closely resembling *T. evansi* in the blood of cattle suffering from a disease known as 'nagana'. He also showed that flies of the genus *Glossina* were associated with the occurrence of 'nagana'. Plimmer and Bradford (1899) later named the parasite *Trypanosoma brucii* (later *T. brucei*). Other species of pathogenic trypanosomes were also isolated from cattle. Ziemann (1905), in the former German Cameroon described an actively motile trypanosome from the blood of domestic ruminants which was later named *T. vivax.* A parasite found in donkey and sheep in Congo (Zaire) by Broden (1904) which was smaller in size than those hitherto described and lacked a free flagellum was duly named *T. congolense.* Later, similar parasites were observed in cattle and

dromedaries in the Congo (Broden, 1906). The important discovery that the pathogenic African trypanosomes undergo a definite cycle of development in the tsetse vector was made by Kleine (1909).

Despite concerted international efforts to control the disease, trypanosomiasis remains a major constraint to livestock production in tsetse infested areas. Approaches to control the disease presently involve chemotherapy or chemoprophylaxis directed against the trypanosomes and measures aimed at reducing or eliminating the vectors. Chemotherapy and chemoprophylaxis of animals at risk are successful but expensive control measures (Dwinger, 1985). Trypanocidal drugs are few and their continued use for many years has, not suprisingly, led to the appearance of drug resistant strains of trypanosomes (Williamson, 1980; Holmes and Scott, 1982) to all known trypanocides including the latest compound used in the field, isometamidium chloride (Bourn and Scott, 1978; Küpper and Wolters, 1983; Pinder and Authie, 1984). In addition to the problems caused by drug resistance, trypanosomes occupy privileged sites including the central nervous system where they are often inaccessible to the action of trypanocidal drugs (Poltera, 1983; Rudin, Pongponratn and Jenni, 1984) and could therefore form foci from which relapse infections might later arise.

Vector control is the major control measure currently being undertaken. Successful eradication programmes have been applied using insecticide spray of tsetse habitats in several African countries such as Nigeria (Spielberger, Na'isa and Abdurahim, 1977), South Africa, Zimbabwe (Chapman, 1976) and Botswana (Davies and Bowles, 1979). Traps and targets impregnated with chemoattractants and insecticides are also being widely used (Hargrove and Vale, 1979; Vale, 1980). However, the presence of vast infected areas and the presence of more than 30 different species of tsetse adapted to varying climatic conditions and vegetation (Ford, 1970) coupled with other economic, environmental and ecological constraints make the feasibility and efficacy of control difficult (Jordan, 1974). Insecticide spraying

and bush clearing to remove tsetse are very expensive undertakings and are really only effective in geographically restricted areas (Akol, 1985). In addition, cleared areas have to be vigorously defended against possible reinvasion (MacLennan, 1981) and effective land-use programmes must be implemented to ensure successful maintenance of cleared areas. Other environmental problems which arise from the effects of spraying insecticides are high mortality rates in insects other than *Glossina*, in insectivorous animals and in some aquatic fauna (reviewed by MacLennan, 1981). As an alternative method of tsetse control, several attempts have been made using biological methods through the release of sterile males (Simpson, 1958; Knipling, 1963; Dame and Schmidt, 1970; Williamson *et al.*, 1983). The males which are sterilized by irradiation compete with wild male tsetse for females. The female tsetse fly mates only once in her lifetime (Glasgow, 1970) and so when mated by an irradiated, sterile male she fails to conceive. This approach is expensive (Roelants and Williams, 1982) and can only be used effectively in areas where the tsetse population has been reduced using other methods such as insecticide spraying. There is also the possibility of increasing transiently the incidence of disease by the release of large numbers of sterile males since they are capable of cyclical transmission (Moloo, 1982).

Immunoprophylaxis against trypanosomes by development of vaccines has been hindered by the ability of trypanosome to undergo variation of the antigen composition of their surface coat. The variable surface glycoproteins (VSGs) which constitute the surface coat of trypanosomes are potent immunogens (Cross, 1978) but the immunity they confer on the host is specific only against variable antigen types (VATs) of trypanosomes bearing identical VSG antigens (Doyle, 1977; Cross, 1978). However, immunity that develops following cyclically-transmitted *T. congolense* infection and treatment with the trypanocidal drug Berenil remains effective against all metacyclic VATs (M-VATs) transmitted by tsetse (Nantulya, Doyle and Jenni, 1980b). Such immunity, however, is still restricted: in this instance it is effective only

against trypanosomes expressing the same M-VAT repertoire or serodeme. Although the M-VAT repertoire is more conserved (Crowe *et al.*, 1983) than that of bloodstream forms, production of a vaccine against this infective stage is hampered by the large number of serodemes present within the various trypanosome species which occur in the field.

There is potential in the use of livestock which are resistant to trypanosomiasis (trypanotolerant) as one of the measures to control disease and enhance productivity in tsetse infested areas. Trypanotolerant animals include the N'dama, Muturu and other West African shorthorn taurine breeds of cattle (Pierre, 1906). Trypanotolerance has also been described among different breeds of indigenous sheep and goats in both East (Griffin and Allonby, 1979) and West Africa (ILCA, 1979). Trypanotolerant animals are often able to survive infection without the use of trypanocidal drugs and remain as productive as other breeds (Roberts and Gray, 1973; ILCA, 1979; Murray *et al.*, 1981). However, the population of trypanotolerant cattle is low and restricted to West Africa and it will need considerable sociological changes as well as enormous financial commitment to introduce them into the vast areas presently at risk from trypanosomiases. Trypanotolerance might have a genetic basis brought about by natural selection during constant exposure to infection over several generations (ILCA, 1979). N'dama and Muturu cattle are also less susceptible to other diseases such as heartwater, anaplasmosis, babesiosis (Epstein, 1971), streptothricosis (Coleman, 1967) and helminthiasis (Murray, Morrison and Whitelaw, 1982). Similarly, certain species of wild bovidae have innate resistance to various diseases of domestic livestock (Murray *et al.*, 1982).

It is possible that in trypanotolerant animals, both immunological and non-immunological factors may affect trypanosome growth and differentiation *in vivo* (Murray *et al.*, 1982). Significantly higher levels of the enzyme polyamine oxidase (PAO) are present in the serum of resistant animals (Roelants, 1986). Dying trypanosomes release a substance known as spermidine which is oxidized by PAO to

produce antibody independent trypanolytic products (Ferrante, Allison and Hirumi, 1982). However this effect alone is probably not sufficient to suppress trypanosome growth and prevent infection. Further, it is expected to be more effective after production of antibodies. Trypanotolerance might also be due to immunological mechanisms which operate more effectively in resistant than in susceptible animals (Murray *et al.*, 1982; Akol *et al.*, 1986a). A more effective and earlier immune response in resistant animals would enable them to control parasitaemia and reduce pathological damage. Differences in immunological responses to trypanosomes have been demonstrated between trypanotolerant and trypanosusceptible breeds of cattle. N'dama cattle with previous experience of trypanosomiasis are able to eliminate trypanosomes more rapidly than susceptible Zebu following secondary trypanosome challenge and thus appear to show a superior secondary immune response (Desowitz, 1959). Shapiro and Murray (1982) demonstrated an association between the greater capacity of N'dama cattle to control *T. brucei* infection and their ability to recognize at least one of three common trypanosome antigens of molecular weights, 110, 150, 300 kilodaltons compared to Zebu.

Following bites by infected tsetse flies, a local skin reaction develops in the host several days later. This reaction is a focus of intense trypanosome multiplication prior to dissemination into the bloodstream. It is possible that the factors which regulate parasite growth and differentiation could operate in the skin and might be important in determining susceptibility of the host (Murray and Trail, 1984). Trypanotolerant cattle as well as wild animals such as buffalo, oryx, eland and waterbuck which show low parasitaemia and only mild clinical disease develop skin reactions at sites of infected tsetse bites which are smaller in size compared to susceptible animals. They also take a longer time to develop parasitaemia than susceptible animals (Murray *et al.*, 1981; Dwinger, 1985; Akol *et al.*, 1986a). The focus of trypanosomes in the skin represents the first host-trypanosome interaction and probably determines the subsequent direction of infection (Gray and Luckins,

1980; Akol and Murray, 1983; Luckins and Gray, 1983; Dwinger, Rudin and Murray, 1988). A number of studies on immunity in tsetse transmitted *T. congolense* infections have shown that protective immunity might operate at the level of the skin (Akol and Murray, 1985; Dwinger *et al.*, 1988). Furthermore, concurrent trypanosome infections interfere with and delay establishment in the skin of a second heterologous serodeme, delay the onset of humoral antibody responses and impair the development of immunity (Luckins and Gray, 1983; Dwinger *et al.*, 1989).

Little work has been done to determine the cellular changes involved in these early host responses. Characterization of cellular subpopulations and their interactions during this early stage of infection could lead to a greater understanding of the immune response, and the basis of trypanotolerance and interference. Until recently lack of cell typing reagents had limited detailed analysis and interpretation of these cytological changes and differences in ruminants. However, the development of hybridoma technology and monoclonal antibodies (MAbs) to bovine leucocyte surface antigens (Baldwin *et al.*, 1986; Ellis *et al.*, 1986; Davis *et al.*, 1988) has helped in identification of phenotypically and functionally distinct leucocyte subpopulations. Attempts have been made therefore to identify cell populations involved in the immunological control of the disease by trypanoresistant animals. Ellis *et al.* (1987) carried out a comparative study of the circulating leucocyte populations in both the trypanotolerant N'dama and susceptible Boran (zebu) breeds of cattle exposed to tsetse-transmitted challenge with *T. congolense*. They observed significant differences in the changes in the cellular dynamics in infected N'dama and Boran cattle. N'dama had consistently higher levels of B cells and total white blood cells compared to the Boran, although the patterns of changes in the two groups were similar. Williams *et al.* (1989) found that following secondary challenge, immune N'dama cattle showed an increase in CD4-positive T cells in contrast to the decrease in T cells observed both in naive and susceptible Borans. Although their studies revealed some differences in peripheral blood cellular responses they did not attempt

to characterize the cells involved at the level of trypanosome growth regulation in the skin and associated lymphoid tissue which might have direct bearing on immunity and susceptibility.

The work described in this thesis investigates the host-trypanosome relationship with emphasis on the pathogenesis and cellular responses associated with local skin reactions which develop in sheep at sites of intradermal inoculation of culture-derived metacyclic forms of *T. congolense*. A panel of monoclonal antibodies specific for various ovine leucocyte antigens (Mackay *et al.*, 1985; Maddox, Mackay and Brandon, 1985; Hopkins, Dutia and McConnell, 1986; Mackay, Maddox and Brandon, 1986) were used in immunocytochemical techniques to characterize and determine the dynamics, response and phenotypes of cells present in the skin reaction, draining peripheral lymph, regional lymph nodes, efferent lymph and in peripheral blood. Transmission electron microscopy was used to study cellular events in the skin prior to development of skin reaction and to investigate the interaction of trypanosomes and cells of macrophages/veiled cell lineage in the peripheral lymph draining from the local skin reactions.

CHAPTER TWO
REVIEW OF THE LITERATURE

2.1 Classification and Nomenclature of Trypanosomes

Trypanosomes are digenetic, eukaryotic organisms which are found in the bloodstream and tissues of vertebrates and in the gut of leeches and arthropods (Hoare, 1972). The systematic classification is shown in Table 2.1.

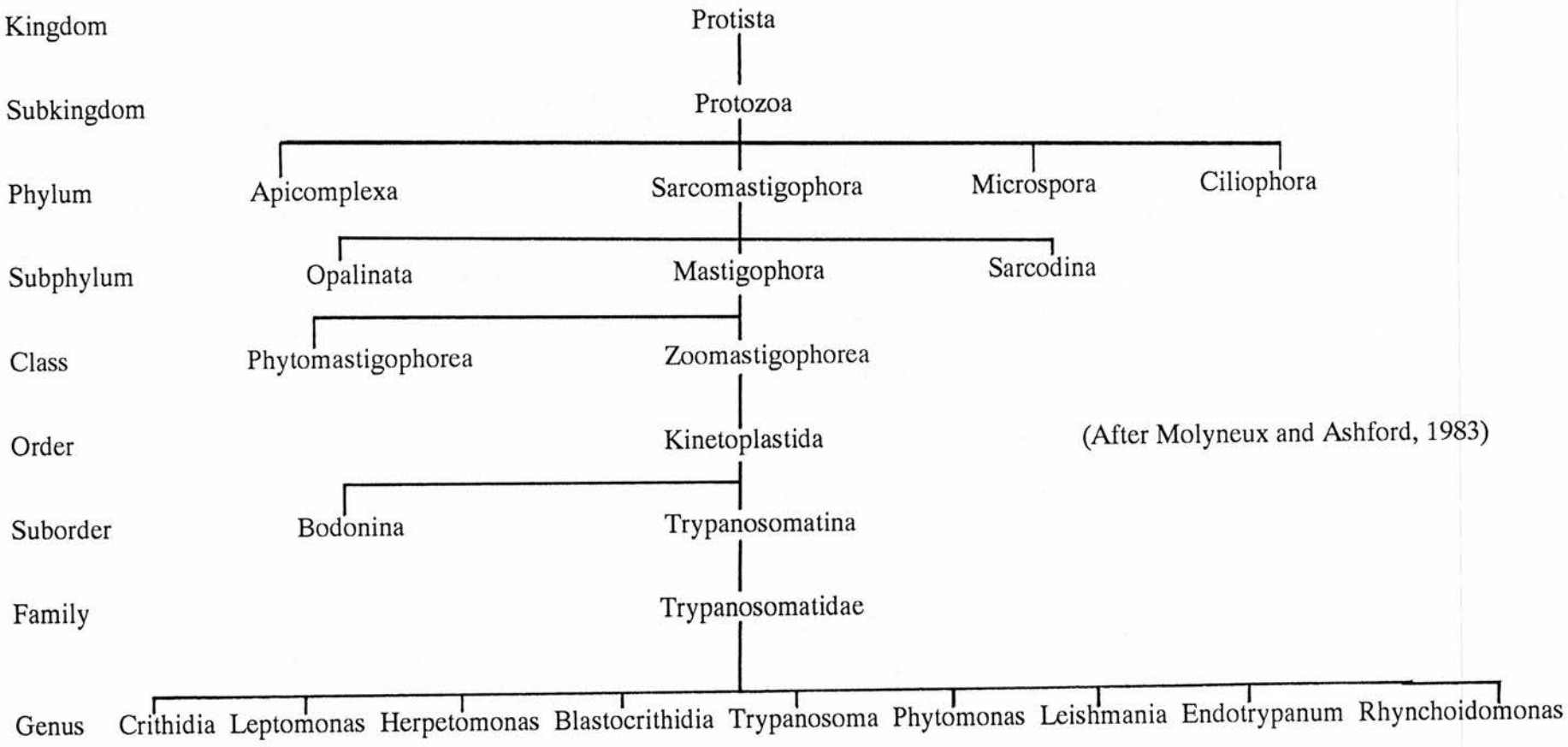
2.1.1 Genus *Trypanosoma* (Gruby, 1843)

The members of this genus are elongated, spindle-shaped or lanceolate protozoa which are 8.0 to 39 μ long. They possess a flagellum which arises at the posterior end of the trypanosome from a basal body at the foot of the flagella pocket. This flagellum runs to the anterior end of the body and is attached along its length to a pellicle to form an undulating membrane. The flagellum may continue forward as a free anterior flagellum in some species. In a stained specimen, a single centrally placed nucleus can be seen. Adjacent to the flagella pocket is a small structure, the kinetoplast, which contains the DNA of the single mitochondrion. Stages found in the mammalian host and the infective stages of the trypanosome in the vector have a 12 to 15 nm thick surface coat which overlies the three-ply plasma membrane as seen in electron micrographs of trypanosome sections (Vickerman, 1969; Steiger, 1973; Vickerman *et al.*, 1980; Gardiner *et al.*, 1986).

2.1.2 Speciation of trypanosomes

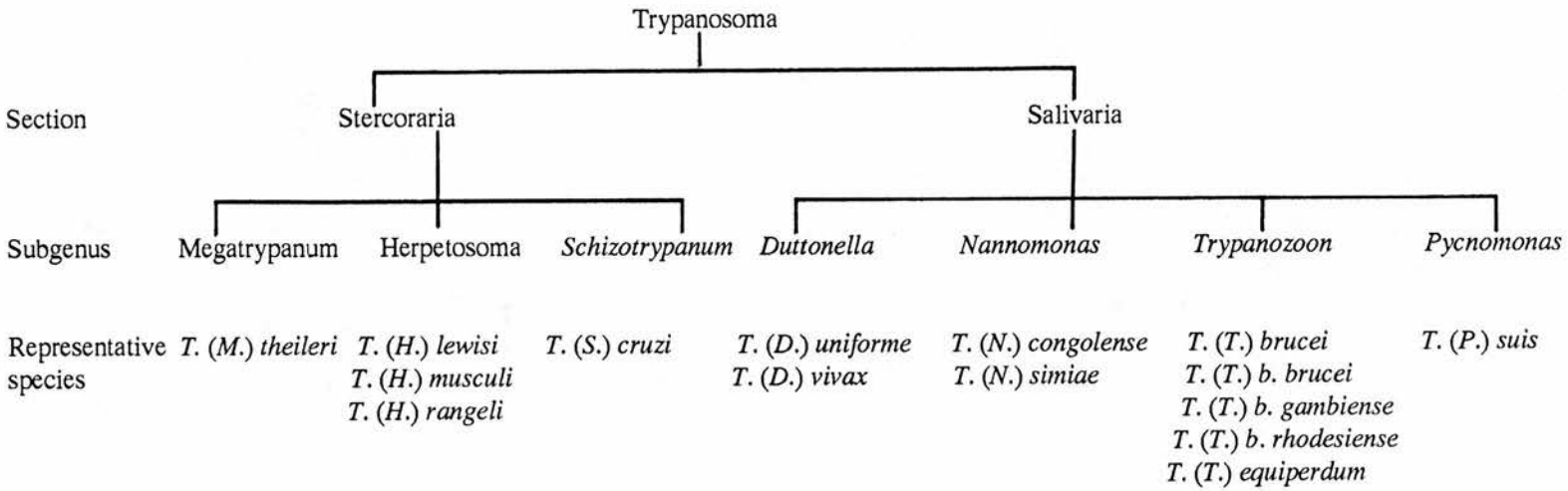
The detailed speciation of members of the genus *Trypanosoma* is given in Table 2.2. Trypanosomes have been divided into two Sections on the basis of the site of development in the insect vector and subsequent method of infection of the mammalian host (Hoare, 1964). Trypanosomes of the Section Stercoraria develop in the terminal gut or posterior station of the insect vector. Transmission to the susceptible hosts is effected by contamination of abraded skin or mucous membranes of the hosts with faeces containing infective forms of the trypanosomes. One species of pathogenic importance in this Section is *Trypanosoma* (*Schizotrypanum*) *cruzi*, which is found in Central and South America and is the causative agent of Chagas' disease or American trypanosomiasis of man. Two other species in this section; *T.* (*Megatrypanum*) *theileri* and *T.* (*M*) *melophagium*, have a cosmopolitan distribution

TABLE 2.1 Classification of kinetoplastida



(After Molyneux and Ashford, 1983)

TABLE 2.2 Classification of the genus *Trypanosoma* (after Molyneux and Ashford, 1983)



and are parasites of cattle and sheep respectively. Although there is no conclusive evidence that these two species are pathogenic (Hoare, 1972) they are important in serological diagnosis where their presence could cause cross-reactivity with pathogenic trypanosomes.

The infective stages of trypanosomes of the Section Salivaria complete their development in the mouthparts or salivary glands (anterior station) of the invertebrate hosts. Transmission occurs when the vector deposits saliva containing infective trypanosomes into the skin of the host when taking a blood meal. African trypanosomes fall within this section and are further classified into four subgenera on the basis of morphology of the bloodstreams forms of the parasites (Table 2.3) and the site of final development in the mouthparts of insect vector. Trypanosomes of the subgenus *Duttonella* comprising *T. (D) vivax* and *T. (D) uniforme*, develop in the proboscis while members of the subgenus *Nannomonas*, comprising *T (N) congolense* and *T (N) simiae*, develop in the midgut and proboscis of the insect vector. Trypanosomes of the subgenus *Trypanozoon* develop both in the midgut and salivary glands of the vector and include *T (T) brucei brucei, T. (T) b. gambiense*, and *T. (T) b. rhodesiense*. Two other species belonging to the subgenus *Trypanozoon, T. (T) evansi and T. (T) equiperdum* do not undergo development in the invertebrate host. *T. (T) evansi* is transmitted mechanically to susceptible host through contaminated mouthparts of biting flies of the genera *Tabanus*, *Chrysops*, *Haematopota* and *Stomoxys* (Vickerman, 1976). *T. (T) equiperdum* does not involve an intermediate host and is transmitted during coitus. *T. (Pycnomonas) suis*, the type species of the subgenus *Pyconmonas*, develops in the midgut and salivary glands of the insect vector.

Different subgenera of African trypanosomes are distinguished microscopically by their morphological appearance (Table 2.3). However, there are no characteristic morphological differences between different species of the same subgenus and speciation was originally based on host specificity and pathogenicity

TABLE 2.3 Some morphological differences of African trypanosomes

			Morphological Characteristics		
Subgenera	Species	Size	Kinetoplast	Free flagellum	Undulating membrane
Duttonella	*T. vivax* *T. uniforme*	18 - 31 μ	Kinetoplast Large and terminally placed	Present in all stages	Inconspicuous
Nannomonas	*T. congolense* *T. simiae*	8 - 24 μ	Kinetoplast medium and marginal and subterminal	Absent in all stages	Inconspicuous
Trypanozoon	*T. brucei brucei* *T.b. rhodesiense* *T.b. gambiense*	17 - 27 μ	Kinetoplast small, subterminal	Present in all stages except infective forms	Conspicuous
Pycnomonas	*T. suis*	9 - 19 μ	Kinetoplast small, sub-terminal and marginal	Present in all stages	Inconspicuous

(Table 2.4) (Hoare, 1972). Biochemical differences in electrophoretic patterns of various isoenzymes (Godfrey and Kilgour, 1976; Gibson, Marshall and Godfrey, 1980) and restriction endonuclease analysis of mitochondria DNA (kinetoplast DNA) have been used to characterize species of the *T. brucei* group (Borst *et al.*, 1980; Borst *et al.*, 1981). Recently, species-specific DNA probes (Massamba and Williams, 1984; Gibson, Dukes and Gashumba, 1988), and species-specific monoclonal antibodies (Nantulya *et al.*, 1987) have been developed which are likely to provide more precise techniques for speciation and subspeciation within the different subgenera of trypanosomes.

2.2 *Glossina*: Vectors of Salivarian Trypanosomes

African pathogenic trypanosomes are transmitted by flies of the genus *Glossina*. These vectors are found exclusively in Africa between latitude 15°N and 28°S. Within these latitudes, it is impossible to rear cattle in many areas, due to the presence of tsetse flies (Figure 2.1). More than 30 different species and subspecies of tsetse have been recognized (Glasgow, 1970). Within a tsetse infested area, many species have a limited local distribution. Three major groups of tsetse flies - *fusca, morsitans* and *palpalis* - are recognized. Flies of the *fusca* group are found in humid and forested areas while members of the *morsitans* group are usually found in woodland or drier savannah vegetation. The *palpalis* group comprises riverine species which are usually found in waterside habitats.

Flies of the *morsitans* group are the major vectors of trypanosomes pathogenic to domestic animals. Since their habitat is suitable for raising livestock, they feed readily on cattle, sheep and goats. The *palpalis* group also presents a threat to livestock due to their proximity to grazing and watering areas. The forest species are efficient vectors of trypanosomes but are rarely in contact with domestic animals (Jordan, 1986).

Both male and female tsetse flies are haematophagous and are totally dependent on bloodmeals. They feed by penetrating the skin of the host using their

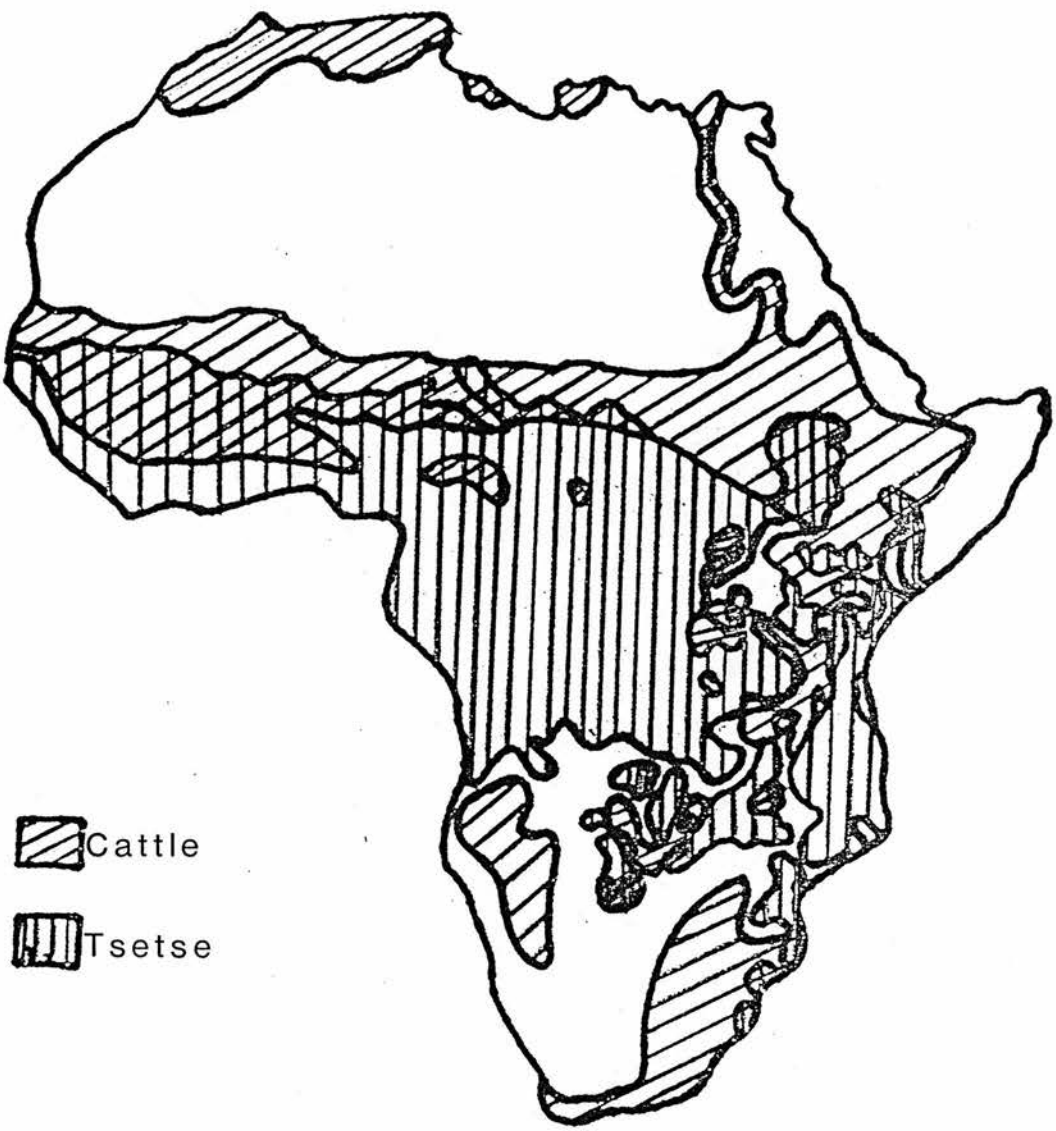
TABLE 2.4 Susceptibility of animals to African trypanosomes

Parasite	Bovines	Sheep	Goats	Equines	Dogs	Pigs	Camels
T. brucei	**	*	*	**^{d}or***^{h}	***	*	***
T. congolense	** or ***	*	*	*	*or***	*	*
T. evansi	**or--	*	*	*^{d}or***^{h}	**	*	***
T. simiae	R	**	***	R	R	****	*or***
T. suis	R	R	R	--	--	***	--
T. vivax	**or***	**	***	*or***	R	R	*or***

**** = peracute, *** = acute, ** = chronic, * = mild, -- = not well known, R = refractory
d = donkeys, h = horses

FIGURE 2.1 Map of Africa showing the distribution of tsetse flies and areas in which cattle are raised.

2.1



specialized piercing mouthparts to create a pool of blood from which they feed. Blood is sucked through the food canal after being mixed with saliva containing an anticoagulant from the common duct of the long salivary glands (hypopharynx). During feeding, an infected tsetse fly deposits saliva containing infective trypanosomes into the skin of the host.

2.3 *Trypanosoma (Nannomonas) congolense*

2.3.1 Morphology and identification

Trypanosoma (Nannomonas) congolense (Broden, 1904) is a small monomorphic parasite ranging between 8 to 24 μ in length (Hoare, 1959). It is devoid of a free flagellum, possesses a medium sized, marginal kinetoplast and an inconspicious undulating membrane. The fine structure of *T. congolense* resembles that of other trypanosomes (Vickerman, 1969). Bloodstream forms of the other member of the subgenus, *T. (N) simiae*, are larger (17.0 to 18.2 μ) and polymorphic (Vickerman, 1969). The nomenclature of *T. congolense* was confusing due to the multiplicity of species names ascribed, based on variations in size and host species infected (Hoare, 1972). Subsequently, a number of revisions of the subgenus excluded all the other "species" but *T. congolense* and *T. simiae* (Hoare, 1972).

More recently, phenotypic and genotypic heterogeneity has been demonstrated among stocks of *T. congolense* isolated from various parts of Africa using DNA probes (Majiwa *et al.*, 1986) and differences in isoenzyme electrophoretic patterns (Young and Godfrey, 1983; Gashumba, Baker and Godfrey, 1988; Knowles *et al.*, 1988). The distribution of distinct enzymic variants (zymodemes) and karyotypes corresponds with different geographical and ecological zones. Similarly, *T. simiae* specific DNA probes have been described (Majiwa and Webster, 1987) which can distinguish this species from *T. congolense*. These molecular and biochemical tools will therefore be useful in accurate intraspecific and specific identification within the subgenus *Nannomonas* for both taxonomic and epidemiological applications.

2.3.2 Life cycle and transmission

2.3.2.1 Development of *T. congolense* in *Glossina* Species

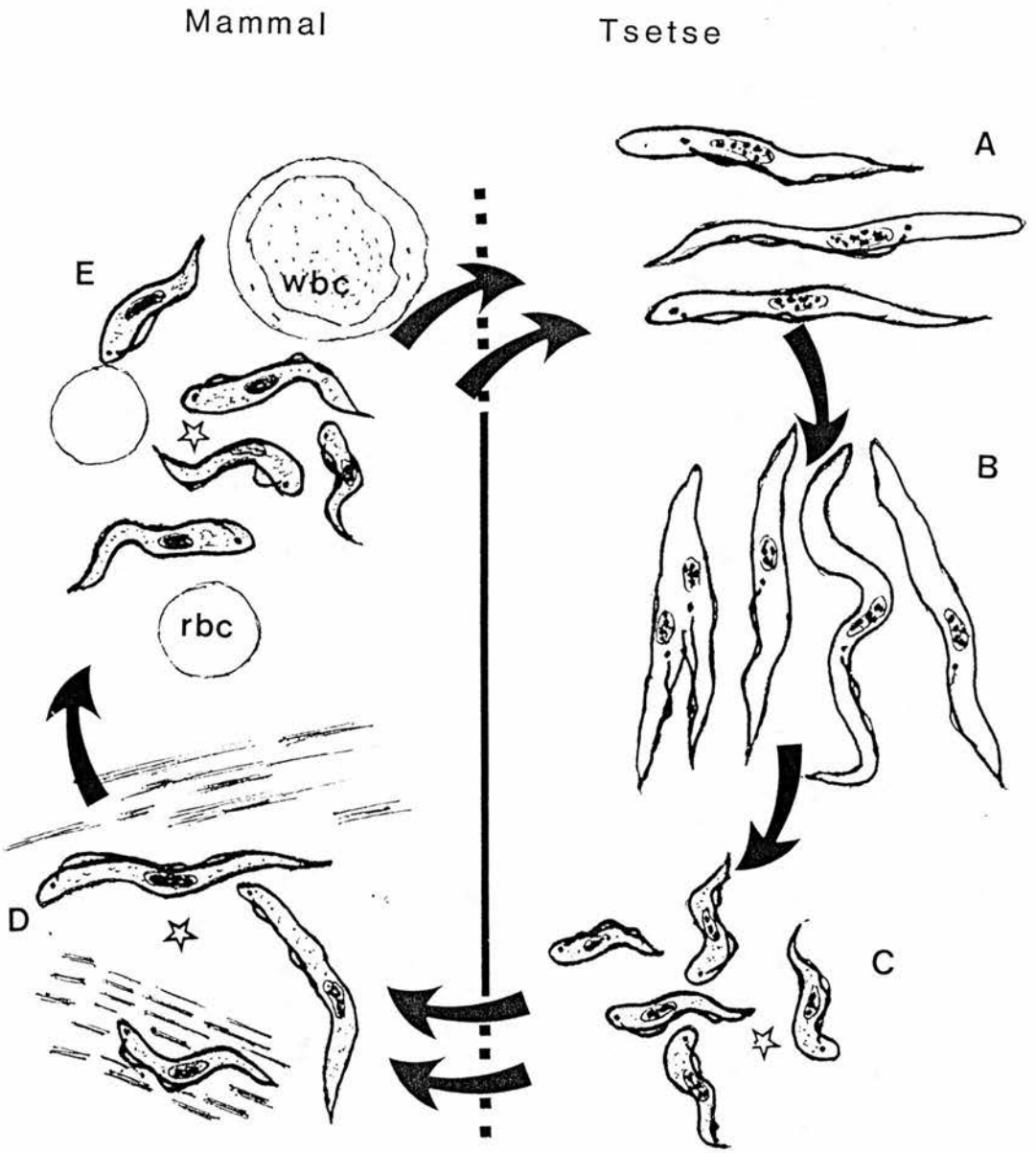
The cycle of development of *T. congolense* in the tsetse fly has been studied by several workers using both light and electron microscopy (Lloyd and Johnson, 1924; Hoare, 1972; Evans *et al.*, 1979; Kaddu and Mutinga, 1980; Thevenaz and Hecker, 1980). A diagramatic representation of the life cycle is given in Figure 2.2. Trypanosomes ingested by the fly in the bloodmeal begin their development in the endoperitrophic space of the midgut. Here, the bloodstream forms lose their surface coats, change their morphology and become elongated trypomastigotes or 'procyclics' which have the kinetoplast positioned close to, but posterior to the nucleus. These forms, which measure more than 40 μ in length, multiply rapidly and then escape through the open posterior end of the peritrophic membrane. They then migrate forward to the proventriculus where they penetrate into the endoperitrophic space and thence proceed via the oesophagus to the proboscis where they attach to the walls of the labrum, and transform into epimastigotes. Epimastigote forms are slender, have the kinetoplast anterior to the nucleus and lack a surface coat. Finally, the epimastigotes transform into infective metacyclic forms which then migrate to the hypopharynx. Metacyclic trypanosomes are morphologically similar to the bloodstream forms, have a surface coat but are non-dividing. The duration of the developmental cycle of *T. congolense* in *Glossina* is variable and ranges between 19 to 53 days (Hoare, 1972) or 7 to 40 days according to Nantulya *et al.* (1978). This variation is probably due to instrinsic biological differences of the trypanosome isolates employed and the species of host the flies are fed on.

2.3.2.2 Development of *T. congolense* in the mammalian host

When a trypanosome-infected tsetse fly bites a susceptible host to obtain a bloodmeal it deposits into the skin saliva containing anticoagulants (Lester and Lloyd, 1928), together with a mixture of infective metacyclic trypansomes and uncoated proventricular and epimastigote forms (Otieno and Darji, 1979). In the skin, the non

FIGURE 2.2 Life cycle of *Trypanosoma congolense* showing the various developmental stages in the tsetse fly and mammalian host. A, Procyclics; B, Epimastigotes; C, infective metacyclic; D, local skin reaction (or chancre) forms; E, Bloodstream forms. Stages C, D and E (stars) possess a surface coat.

2.2



dividing metacyclic forms transform into rapidly dividing 'mammalian forms' before invading the circulatory system. Transitional or intermediate intracutaneous forms have been suggested to occur during transformation of metacyclic to mammalian bloodstream forms (Dwinger *et al.*, 1988). Ultrastructurally, this intracutaneous stage differs from the bloodstream form by lack of lipid inclusions and presence of a well developed secretory reticulum. The mitochondrion is smaller than that of metacyclic forms, probably reflecting differences in the physiological status of the two forms.

Trypanosomes leave the skin, the main developmental site, probably after biological changes associated with maturation to bloodstream forms (Akol and Murray, 1982). Observations of trypanosomes in dermal lymphatics, draining lymph nodes and efferent lymph of cattle and goats indicate that these parasites migrate into the bloodstream through the lymphatic system (Gray and Luckins, 1980; Akol and Murray, 1986; Dwinger *et al.*, 1990). Once in the bloodstream trypanosomes multiply by continuous binary fission (Hoare, 1972). *T. congolense* shows a preferential localization in peripheral blood vessels (Losos and Ikede, 1972; Losos *et al.*, 1973). The parasites attach to walls of capillaries by their anterior ends (Banks, 1978). Subsequent recirculation of trypanosomes seems to be through the blood since very few parasites are detected in the lymph after the initial stages of infection (Akol and Murray, 1986).

2.3.3 Cultivation of *T. congolense in vitro*

Significant progress has been made in *in vitro* culture of African trypanosomes. Most life cycle stages of *T. congolense* and other trypanosome species can now be cultivated *in vitro*). The initial success in the production of culture-derived metacyclic forms of *T. congolense* was reported by Gray *et al.* (1981). Cultures were initiated by placing infected *Glossina* proboscides beside bovine dermal collagen explants. Subpassages of trypanosomes into culture medium without bovine dermal explants resulted in continuous production of metacyclic forms. A simpler culture technique to produce more metacyclic forms was achieved by replacing bovine

dermal collagen explants with commercial purified bovine dermal collagen (Hirumi, Hirumi and Moloo, 1982). Continuous cultures of metacyclic forms have been maintained in Eagles minimum essential medium supplemented with 20% fetal calf serum in plastic culture flasks in the absence of collagen (Gray *et al.*, 1979; Ross *et al.*, 1985).

Culture-derived metacyclic forms of *T. congolense* cause infection in cattle (Akol *et al.*, 1985) and rabbit (Luckins, Rae and Gray 1981) and also induce local skin reactions at inoculation sites similar to those elicited by infected tsetse fly bites. In addition, they retain the antigenic characteristics of the metacyclic trypanosomes from which they were derived. Such *in vitro* culture systems therefore provide reliable sources of large numbers of metacyclics which retain the morphological, biological and antigenic characteristics of trypanosomes found in the tsetse fly and mammalian hosts and provide defined organisms for immunological and biochemical studies (Luckins *et al.*, 1981; Crowe *et al.*, 1983; Gray *et al.*, 1984). The use of culture-derived metacyclic forms of *T. congolense* to study the pathological and immunological nature of local skin reactions has its advantages. The dose of trypanosome used to infect the animals can be standardized. It also offers flexibility in the choice of inoculation sites on the host.

2.3.4 Pathogenesis of the disease in domestic ruminants

2.3.4.1 Clinico-pathological features of *T. congolense* infection

T. congolense is the most important cause of animal trypanosomiasis in East Africa and second only to *T. vivax* in West Africa (Fiennes, 1950; Mulligan, 1970). Pathogenicity is dependent upon the particular isolate of *T. congolense* due to the wide variation in virulence (Fiennes *et al.*, 1946; Griffin and Allonby, 1979). The disease affects mainly cattle although sheep,goats and dogs are also affected.

Severity of the disease in ruminants varies considerably. Acute, subacute and chronic forms have all been described (Fiennes, 1954; Losos *et al.*, 1973; Griffin and Allonby, 1979). A chronic form of the disease lasting longer than three months is

more common (Morrison Murray and McIntyre, 1981). The prepatent period of *T. congolense* infection varies, ranging from nine to 25 days in sheep (Griffin and Allonby, 1979) and about 15 days in cattle. Clinical signs observed include pallor of mucous membranes, intermittent fever, enlargement of superficial lymph nodes, progressive but gradual loss of body condition, decreased fertility and abortion (Naylor, 1971; Wellde *et al.*, 1974; Griffin and Allonby, 1979). The major pathological signs of the disease are anaemia, pale parenchymatous organs, enlarged lymph nodes, haemolymph nodes, spleen and liver and inflammatory changes in several organs including the heart, testicles, lungs and kidneys (Losos *et al.*, 1973; Kaliner, 1974; Griffin and Allony, 1979; Valli and Forsberg, 1979; Kaaya and Oduor-Okelo, 1980).

Progressive anaemia is the most significant clinical manifestation of African trypanosomiasis in domestic livestock (Murray, 1979; Morrison *et al.*, 1981) and in infections with *T. congolense* in Zebu cattle is probably a primary cause of death (Mamo and Holmes, 1975). There is a progressive decrease in the number of red blood cells and the packed cell volume (PCV) (Morrison, Murray and Akol, 1985) which correlates with the level and duration of parasitaemia and is dependent on the serodeme of the infecting trypanosome. The aetiology of anaemia in trypanosomiasis is thought to be an interplay of several factors: haemolysis, haemodilution, decreased erythropoiesis and/or non-compensatory erythropoiesis (Jenkins and Facer , 1985) all of which might play a part during the course of infection.

Initially, the anaemia is haemolytic due to an increased rate of red cell destruction (Dargie *et al.*, 1979; Murray *et al.*, 1980; Saror, 1980). This is dependent upon the level of parasitaemia since treatment with trypanocidal drugs usually results in dramatic recovery (Holmes and Jennings, 1976). Histological evidence of haemolysis, namely erythrophagocytosis in the spleen, liver and lymph nodes, together with hemosiderin deposition have been described in sheep infected with *T. congolense* (Mackenzie and Cruickshank, 1973). This red cell destruction and tissue

damage in sheep infected with *T. congolense* is probably mediated primarily by biologically active substances released by dying trypanosomes (Huan *et al.*, 1975; Tizard *et al.*, 1978b) circulating immune complexes (Mackenzie and Cruickshank, 1973; Kobayashi, Tizard and Woo, 1976) or by the activated mononuclear phagocytic system (MPS) (Murray *et al.*, 1974a). Later in infection, the parasitaemia declines to almost undetectable levels but red cell destruction continues and animals are refractory to trypanocidal therapy. This has been attributed to progressive dyshaemopoiesis and erythrophagocytosis by the activated MPS (Dargie *et al.*, 1979; Murray *et al.*, 1980). Finally, in animals at late stages of chronic infection, anaemia is due to failure of the bone marrow to reutilize and incorporate iron to developing red blood cell precursors, resulting in a decrease in production of red cells (Dargie *et al.*, 1979).

2.4 Antigenic Variation in African Trypanosomes

African trypanosomes have evolved various ways of coping with their host's defence systems (De Lange, 1986). The primary mechanism employed to evade the host's immune responses is by the generation of antigenically distinct types during chronic infections. This process, known as antigenic variation, is manifested by the sequential expression of variant surface glycoproteins (VSGs) which form a dense homogenous layer of surface coat covering the trypanosome body and flagellum (Vickerman, 1969, 1978; Cross, 1975; 1990). The surface coat is a unique morphological feature of the bloodstream and infective insect stages of all salivarian trypanosomes (Vickerman, 1969, 1978). VSGs account for almost 10% (10^7 molecules per cell) of the total protein of the bloodstream forms of *T. brucei* (Cross, 1975).

The VSG is highly immunogenic, and the antigenic determinants within the molecule are responsible for the induction of variant-specific immunity (Cross, 1975). When an immune response is elicited in the host against the infecting trypanosome variable antigen types (VATs), the parasite is able to express an alternative and

immunologically distinct VSG (Cross, 1978). This results in the continuous generation of different VATs and the setting up of persistent chronic infections (Van Meirvenne *et al.*, 1975). Antigenic variation may be recognized as a series of sequential changes in the serological characteristics of surface coat antigens during the course of infection (Cross, 1978). Clinically, in infected hosts this is seen as fluctuating waves of parasitaemia with each peak representing a predominant or prevailing VAT (Vickerman, 1978). Immunological destruction by the host of trypanosomes expressing earlier VATs and the reappearance of new VATs are responsible for the waves of parasitaemia observed in trypanosomiasis (Esser, Schroenbechler and Gingrich, 1982). The number of switched trypanosomes in the population at a particular time has been estimated to be between 10^{-4} and 10^{-5} (Van Meirvene *et al.*, 1975; Doyle, 1977). Analysis of switching from a defined VSG type to another type during *in vitro* cultivation of *T. brucei* suggested frequencies ranging from 3.5×10^{-6} to 1.4×10^{-7} (Lamont, Tucker and Cross, 1986).

Antigenic variation stops during transformation from bloodstream to procyclic forms *in vitro* or in the midgut of the tsetse fly (Ehlers, Czichos and Overath, 1987). However, VSG expression starts in the tsetse salivary gland (in the case of *T. brucei*) after cessation of cell division (Tetley *et al.*, 1987). The metacyclic population is antigenically heterogenous and predictable although comprising only a small proportion of the entire VAT repertoire (Le Ray, Barry and Vickerman, 1978). One strain of *T. congolense* contains only 12 metacyclic VATs (M-VATs) and in *T.b. rhodesiense* the repertoire is also limited (Crowe *et al.*, 1983; Barry, Crowe and Vickerman, 1983; Turner *et al.*, 1988). Metacyclic VSGs of *T. brucei* are expressed for only a short period of time after transfer to the mammalian host (Esser and Schrenbechler, 1985; Barry *et al.*, 1983). In contrast *T. congolense* M-VATs continue to be expressed in the animal although new VATs are thought to be generated within the local skin reaction (Luckins *et al.*, 1990). M-VATs are re-expressed later in infections initiated by bloodstream forms of *T.b. rhodesiense* (Barry *et al.*, 1983), *T.*

congolense (Nantulya *et al.*, 1984) and *T. vivax* (Nantulya, Musoke and Moloo, 1986). This is an indication that the M-VAT expression is not restricted to metacyclic trypanosomes alone and that the bloodstream forms are capable of re-expressing VATs which are shared or related to metacyclic forms.

2.4.1 Variant surface glycoprotein

The purified form of VSG consists of glycoproteins of molecular weight of 60 to 65 kilodaltons (Cross, 1975; Cross and Johnson, 1976). *T. brucei* VSGs contain between 433 to 470 amino acids (Allen Dickens and Cross, 1982; Boothroyd *et al.* 1982) while that of *T. congolense* is shorter containing only 372 amino acid residues (Strickler *et al.*, 1987). VSG also contains 7 to 17% carbohydrate by weight (Johnson and Cross, 1977). Cleavage of glycoproteins using trypsin has shown that it contains two 'domains' (Johnson and Cross, 1979). The amino (N-) terminal domain is the exposed two-thirds of the VSG sequence which is released into solution on trypsin treatment of trypanosomes. The other third comprises the carboyxl (C-) terminal domain which is membrane orientated and contains most of the carbohydrate. This domain remains attached to the trypanosome following trypsin treatment unless released by cell rupture (Cross and Johnson, 1976).

The N-terminal amino acid sequence is hypervariable and determines the antigenic characteristics of the VSGs (Miller *et al.*, 1984) while the last 110 amino acids at the C-terminus show some conservation (Boothroyd *et al.*, 1981; Matthyssens *et al.*, 1981). Sequence homologies of VSGs are detectable up to about 50 amino acids from the C-terminus. The sequences then diverge to such an extent that over the remaining 400 amino acids, few homologies are detectable other than the placement of cysteine residues (Allen *et al.*, 1982; Pays *et al.*, 1983; Rice-Ficht, Chen and Donelson, 1981). Sequence analysis of cDNA clones have revealed that the VSGs have an N-terminal clevrable hydrophobic sequence that function as a signal peptide (Pays *et al.*, 1983; Rice-Ficht *et al.*, 1981; Boothroyd, 1982). The C-terminus of all VSGs end with either aspartic acid, serine or asparagine residues all of which have

potential for N-glycosylation sites (Holder and Cross, 1981). *T. brucei* VSG N-linked glycans contain either glucosamine, mannose and galactose or ethanolamine (Johnson and Cross, 1977; Holder, 1985). *T. congolense* VSG N-linked glycans also contains sialic acid (Rautenberg, Reinwald and Risse, 1981; Savage *et al.*, 1984). In rabbits, the carbohydrate of the VSG is immunogenic and is known as the cross reacting determinant (CRD) as antibody produced cross-reacts with all VSGs (Barbet and McGuire, 1978; Cross, 1979; Holder and Cross, 1981). The CRD is covalently linked to a phosphoglycolipid which contains myristic acid. This glycolipid is thought to provide the lipid anchor of the VSGs to the plasma membrane of the trypanosome (Ferguson and Cross, 1984; Ferguson, Haldar and Cross, 1985). The surface coat forms a compact impermeable barrier against the action of antibody and complement (Johnson and Cross, 1979) by arrangement of a series of secondary and tertiary VSG structures which are cross-linked via oligosacharide bridges. The formation series of α-helical structures strengthens the VSG molecule (Freymann *et al.*, 1984). Analysis of VSG using monoclonal antibodies has revealed the presence of several antigenic determinants. Only a fraction of the VSG variability is expressed in the form of antigenically distinct epitopes on living trypanosomes (Pearson, Pinder and Roelants, 1980; Olenick, Travis and Garson, 1981). These epitopes are thought to be due to protein folding at the N-terminal and are thus conformationally labile (Miller, Allan and Turner, 1984).

2.4.2 Genetic basis of antigenic variation

The switching by trypanosomes from one VSG to another is independent of antibody pressure as demonstrated by occurrence of antigenic variation in *in vitro* cultured bloodstream forms of *T. brucei* and *T. congolense* (Doyle *et al.* , 1980; Luckins *et al.*, 1986). These switches in the type of coat protein synthesized are consequences of sequential activation of as many as 1000 different VSG genes present in the genome of the diploid trypanosomes (Gibson *et al.*, 1980; Borst *et al.*, 1982; Van der Ploeg *et al.*, 1984). Trypanosomes reserve 10% of their genome for

programmed antigenic variation (Borst *et al.*, 1982). VSG genes are either located in chromosome-ends (telomeres) or at chromosome-internal sites (De Lange, 1986). Antigenic variation occurs when a single VSG gene is selected from the large repertoire of the available VSG genes and is selectively transcribed and translated (De Lange, 1986). This is effected by duplication of a 'basic copy' of the VSG gene which is not transcribed, followed by transposition of the new copy, the 'expression-linked copy' (ELC) into a unique transcriptionally active site. These expression sites are all telomeric (De Lange, 1986). The duplicative transposition of chromosome-internal VSG genes is probably due to a gene conversion of a previously expressed ELC by the incoming gene (Pays *et al.*, 1983; Borst and Cross, 1982; Rice-Ficht *et al.*, 1982). Telomeric VSG genes can be activated without duplication or any other DNA rearrangement suggesting that active sites are switched between several expression sites (De Lange, 1986). However, in some cases activation of telomeric VSG genes is due to duplication of one telomere at the expense of another (extensive telomere conversion) (Laurent *et al.*, 1984; Van der Ploeg *et al.*, 1984).

2.5 Immune Responses and Immunity in African Trypanosomiasis

Infection of laboratory animals with African trypanosomes is invariably fatal due to inability of the host to achieve a state of sterile immunity (Murray and Morrison, 1979; Bancroft and Askonas, 1985). In contrast, although domestic ruminants may succumb to infection, they often develop a chronic form of the disease and, occasionally, are able to control the infection and may recover. Thus, the severity and outcome of trypanosome infection depends not only on the virulence of the parasite but also on the comparative resistance of the host. The principal factor which determines the resistance is the effectiveness of the host immune responses. Protective immune responses are directed against the VSG of the surface coat (Murray and Urquhart, 1977). However, these host responses are not fully effective: trypanosomes are able to escape to extravascular sites where they may be less accessible to the effects of hosts protective responses (Seed, 1974) as well as being

able to undergo antigenic variation (Gray, 1985; Vickerman, 1978) thereby establishing persistent infections. The host repeatedly mounts specific protective responses against new VATs throughout infection. This intense stimulation of the immune system is responsible for some of the pathological effects of the disease including anaemia, immunosuppression, autoimmunity, immune complex mediated tissue damage and interference in establishment of superinfections.

2.5.1 Antibody responses to trypanosomes

Susceptibility and survival of trypanosome infected hosts depends on early and continued effective specific antibody responses to successive waves of parasitaemia. For example, C3H/He mice which produce low levels of antibodies are unable to control the initial wave of parasitaemia following *T. vivax* infection and succumb nine to 13 days later. In contrast, C57B1/6 mice are able to limit the initial parasitaemia by the production of high levels of anti-trypanosome antibodies and therefore survive for several weeks (Mahan *et al.*, 1986). Specific humoral antibody responses are evident in rats and rabbits a few days after infection with *T. brucei* (Seed, 1972; Zahalsky and Weinberg, 1976; Seed, 1977). Antibodies are primarily directed against the VSG and are thought to be elicited by VSG bound to trypanosomes rather than by VSGs released by trypanosomes (Black, Hewett and Sendashonga, 1982). Analysis of the induction stimuli of anti-*T. brucei* VSG antibodies in mice revealed that remission of the initial wave of parasitaemia is due to humoral responses against the external determinants of surface expressed VSG (Sendashonga and Black, 1982). In cattle infected with either *T. congolense* or *T. brucei*, protective immune responses correlate well with the levels of anti-trypanosome antibodies (Morrison *et al.*, 1982a; Wells *et al.*, 1982). Specific neutralizing activity is present in both IgM and IgG fractions of serum with maximum activity being present in IgM 17 to 22 days after *T. congolense* infection (Morrison *et al.*, 1982a). Similarly, in cattle infected with *T. brucei* VSG-specific IgM antibodies are more efficient than IgG in neutralizing parasite infectivity (Musoke *et al.*, 1981). Destruction and elimination of trypanosomes from the host is

primarily mediated by parasite-specific antibodies. Trypanosomes exposed to VAT specific antibodies and complement lyse *in vitro* (Lourie and O'Connor, 1936; Van Meirvenne *et al.*, 1975). Complement seems to be a requirement for immune lysis of *T.b. rhodesiense* at low antibody levels (Diggs *et al.*, 1976b; Flemmings and Diggs, 1978) but not at high antibody levels (Balber *et al.*, 1979). Lysis of *T. vivax* can occur in the absence of complement (Clarkson and Awan, 1969) but the reaction proceeds more consistently and rapidly in its presence (De Gee, Shah and Doyle, 1979). It is uncertain which particular complement pathways activated during immune lysis of trypanosomes although most studies indicate that both the classical and the alternative pathway are operative (Flemmings and Diggs, 1978; Balber *et al.*, 1979). There are no conclusive studies indicating the requirement of complement in elimination of trypanosomes *in vivo*. Depletion of complement *in vivo* using cobra venom factor (CVF) does not affect the course of parasitaemia in mice infected with *T. brucei* indicating that complement is not necessary for trypanosome elimination (Shirazi *et al.*, 1980). However, since complement levels are reduced to only 10% rather than eliminated by CVF, this conclusion may not be justified (Vickerman and Barry, 1982).

In the presence of specific antibodies *in vitro*, *T.b. brucei* and *T.b. gambiense* readily attach to and are subsequently phagocytosed by mouse and rat macrophages (Lumsden and Herbert, 1967; Takayanagi, Nakatake and Enriquez, 1974a,b; Stevens and Moulton, 1978; Takayanagi *et al.*, 1987). Attachment of antibody coated trypanosomes to phagocytic cells appears to be via Fc receptors since $F(ab)_2$ fraction of antibody is incapable of effecting binding (Takayanagi *et al.*, 1987). Decomplementation of immune serum does not inhibit attachment *in vitro* (Takayanagi *et al.*, 1974b) indicating that although it is possible that trypanosomes adhere to macrophages via the C3 receptor (Takayanagi *et al.*, 1987) it is not the most important requirement. Trypanosome killing by phagocytic cells might be effected in other ways. Specific antibodies and complement mediate *in vitro* cytotoxic responses of

bovine granulocytes and monocytes and murine peritoneal macrophages against *T. congolense* (Schmitz *et al.*, 1984). Similar observations have been made during interactions of *T. brucei* and mouse peritoneal cells (Rossi and Dean, 1988). Cytolysis of trypanosomes by phagocytic cells *in vitro* appears to occur extracellularly and to precede phagocytosis (Rossi and Dean, 1988). However it is not clear whether such cell-mediated antibody dependent cytotoxicity of trypanosomes occurs *in vivo.*

Phagocytosis by mononuclear phagocytic cells of trypanosomes coated with specific antibodies appear to be a major mechanism of elimination of the parasite *in vivo* (Holmes *et al.*, 1979; MacAskill *et al.*, 1980). Studies on the clearance of ^{75}S-methionine labelled *T. brucei* from immune mice indicated that the MPS system in the liver and lungs was the site of destruction of the trypanosomes (Holmes *et al.*, 1979; MacAskill *et al.*, 1980). Histological and ultrastructural studies of various organs from mice infected with *T. brucei* revealed the presence of numerous macrophages containing phagocytosed parasites (Anosa and Kaneko, 1983; Moulton, 1986).

2.5.2 Cellular immune responses and T cell function in African trypanosomiasis

The significance of cellular immune responses in the development of protective immunity against trypanosome infections has received relatively little attention compared with studies on humoral responses. However, the few studies indicate that cellular responses do not play a major role in survival and immunity to the disease. Athymic or irradiated mice infected with *T. brucei* are able to mount a functional and in some cases an enhanced anti-trypanosomal immune response (Campbell, Esser and Phillips, 1978; Askonas *et al.*, 1979; Clayton, Ogilive and Askonas, 1979). Immunity to trypanosomes is also not adoptively transferable with T cell-enriched spleen cell populations (Takayanagi and Nakatake, 1975; Campbell and Phillips, 1976). Similarly, treatment of immune spleen cells *in vitro* with anti-thymic cell antiserum to remove T cells does not inhibit their capacity to transfer immunity (Takayanagi and Nakatake, 1975).

However, despite the apparent lack of a role in protective response, T cells are responsive to trypanosome antigens following infection or immunization of the host. T cells from mice infected with *T. brucei* or immunized with irradiated *T. rhodesiense* proliferate *in vitro* in presence of trypanosome antigens (Gasbarre, Hug and Louis, 1980; Campbell *et al.*, 1978). Other studies, designed to investigate T cell responsiveness to trypanosome antigens *in vivo* are conflicting: delayed type hypersensitivity (DTH) reactions to trypanosome antigens occur in rabbits infected with either *T. brucei* or *T. rhodesiense* (Tizard and Soltys, 1971). These reactions appear to be directed to common antigens as cross reactions between different trypanosome species occur. Similar reactions have been observed in mice immunized with killed *T. rhodesiense* following injection of trypanosome extracts (Finerty, Krehl and McKelvin, 1978). However, Mansfield and Kreier (1972) found that spleen cells or peritoneal exudate cells from *T. congolense* infected rabbits did not produce lymphokines when exposed to trypanosome antigens *in vitro* and infected rabbits showed no DTH responses when inoculated with trypanosome extracts.

Cellular responses and T cell function in immune responses to African trypanosomes in domesticated animals is not well defined, but, as in laboratory rodents, there is evidence of a T cell response following infection or immunization with trypanosomes. Cattle immunized with formalinized *T. congolense* develop DTH reactions at sites of intradermal inoculation of ultrasonicated homologous *T. congolense* antigen (Emery, Wells and Tenywa, 1980). In the presence of *T. congolense* antigen, peripheral blood leucocytes (PBLs) from these animals proliferated *in vitro*. Similar PBL responsiveness *in vitro* was observed in cells collected from cattle infected with *T. congolense* either during the early stages of infection, prior to development of local skin reaction, or after trypanocidal therapy when trypanosomes were eliminated (Emery *et al.*, 1980c). No such responsiveness was demonstrable in cattle undergoing active infection probably due to depression of T cell functions. PBLs from cattle immunized with *T. brucei* show *in vitro*

proliferative responsiveness in presence of sonicated *T. brucei* antigens (Wells *et al.*, 1982). This response was specific for VSG used for immunization and could not be elicited in animals immunized with purified VSG. T cell responses appear to be dependent on the form in which antigens are presented and processed by accessory cells: it is therefore possible that *in vivo* VSG antigens attached to parasite fragments are more easily presented and recognized by T cells.

Macrophages are important in the initiation and regulation of the immune response in view of their phagocytic activity, their role as antigen presenting cells and their interaction with T and B cells (Unanue, 1981; Nathan, Murray and Cohn, 1980). During trypanosome infections, increased numbers of macrophages are found in many organs (Clayton *et al.*, 1980; Morrison, Murray and Bovell, 1982). Macrophages from *T. brucei* infected mice contain cellular debris (Clayton *et al.*, 1980) and express high levels of Ia antigens, lower levels of Fc receptors, mannose receptors, MAC1 and F4/80 surface markers, all of which are consistent with surface phenotypes of activated macrophages (Grosskinsky *et al.*, 1983). These activated macrophages might serve to present antigens to T cells which in turn influence B cell function and antibody production by elaborating an array of lymphokines (Bancroft and Askonas, 1985).

Infection of mice with African trypanosomes augments production of interferon (IFN) after onset of parasitaemia (Bancroft *et al.*, 1983; De Gee, Sonnenfled and Mansfield, 1985). Two peaks of IFN are produced during the course of infection. The first peak coincides with the first parasitaemic wave and is predominantly of α/β type. The second peak, which is predominantly IFN-gamma (IFN-y) coincides with the period of production of specific antibodies and remission of parasitaemia (De Gee *et al.*, 1985). IFN α/β is probably produced following stimulation of null cells and macrophages (Mayor-Withey *et al.*, 1978; Grosskinsky *et al.*, 1983). IFN-y is a T cell product and might reflect the intense mitogenic pressures on these cells during infection (Mayor-Withey *et al.*, 1978). IFN prevents invasion of

host cells by parasites and elicits intracellular killing of parasites (De Gee *et al.*, 1985) but its role in African trypanosomiasis is unclear. Exogenous IFN does not have any effect on the level of parasitaemia but IFN production in infected mice is highest in resistant strains (De Gee *et al.*, 1985). It is possible that IFN compensates for the non-specific immunosuppressive events by inhibiting secondary bacterial or viral infections in resistant mice.

2.5.3 Induction of protective immunity in African trypanosomiasis

Protective immunity against single VATs, several VATs or a single serodeme of trypanosomes can readily be induced in different hosts. The most commonly used methods of immunization have been establishment of infection followed by treatment with trypanocidal drugs or inoculation of irradiated non-infective trypanosomes (Murray and Urquhart, 1977; Morrison *et al.*, 1982a). Under field conditions, livestock exposed to natural challenge of trypanosomes develop immunity to re-infection, although acquisition of such resistance is dependent on both the degree of trypanosome challenge and efficacy of drug treatment (Whiteside, 1972; Wilson *et al.*, 1975, 1976; Leach and Roberts, 1981). Immunity to bloodstream forms of *T. brucei* and *T. congolense* following syringe inoculation is VAT specific (Hajduk, 1984). Immunization of cattle with 10^7 irradiated non-infective forms of *T. brucei* administered intravenously confers protection against challenge with 10^3 homologous trypanosomes 14 days later (Morrison *et al.*, 1982a). However, immunization using purified VSG is only possible when administered together with Freund's complete adjuvant (Morrison *et al.*, 1982a; Wells *et al.*, 1982). Unlike irradiated or live trypanosomes, purified VSG probably elicits the production of non-protective antibodies directed against epitopes usually masked in live trypanosomes (Morrison *et al.*, 1982a). In mice, protective immunity against intraperitoneal challenge with *T. brucei* of a homologous antigenic type is produced by single doses of a variety of vaccines, including killed organisms, released antigens, formalinized whole infected

blood or plasma administered in crude form, or in water-in-oil or multiple emulsions (Herbert and Lumsden, 1968).

Immunity induced against trypanosomes by infection and trypanocidal drug treatment is directed against VATs which develop during the course of infection (Nantulya *et al.*, 1980b; Luckins, Rae and Gray, 1983; Akol and Murray, 1985). Nantulya *et al.* (1980b) immunized mice by subjecting them to multiple bites by tsetse flies infected with a clone of *T. congolense* over an eight day period and trypanocidal drug treatment 10 days after the first tsetse bite. Mice which received 12 to 15 infective bites on two occasions 21 days apart were protected against homologous challenge. However, immunity elicited was short lived lasting for only seven months. Similar results were obtained in rabbits immunized by cyclical infection and treatment with homidium chloride seven days after development of local skin reaction but in this case immunity lasted for about 10 months (Luckins, Rae and Gray, 1983). Cattle primed by cyclical infection with *G. morsitans morsitans* infected with cloned derivations of *T. congolense* and treated with berenil after 3 to 4 weeks were immune to cyclical challenge with homologous clones 3 to 5 weeks later (Akol and Murray, 1985). However, immunity induced against stocks was not effective when the animals were challenged with the parent stock from which the clones were derived. This indicated that the parent stock contained more than one serodeme and the immunity induced using one clone was serodeme specific. Similar results have been obtained in goats infected with *T. brucei* (Emery *et al.*, 1980), in sheep infected with *T. congolense* (Uilenberg, Maillot and Giret, 1973) and in cattle inoculated intradermally with cultured metacyclic forms of *T. congolense* (Akol and Murray, 1985). Provided the same stock or serodeme is used for primary infection and challenge, the selected host is immune to re-infection.

Animals which recover spontaneously from infection can also develop resistance to subsequent homologous tsetse challenge. Cattle infected intravenously with bloodstream forms of *T. brucei* and *T. congolense* (Nantulya *et al.*, 1980b)

became aparasitaemic 16 to 32 weeks after infection. Sera from these animals were shown by immunofluorescence and neutralization to contain antibodies against all metacyclic VATs of the infecting clones of *T. brucei* or *T. congolense*. These animals were immune to challenge by bites of *G. morsitans morsitans* infected with the same clone or another clone of the same serodeme but they were susceptible to tsetse transmitted heterologous challenge with *T. congolense* or *T. brucei*. These findings suggest that during chronic infections bloodstream trypanosomes are capable of expressing VATs which possess surface epitopes identical to these of metacyclic VATs.

Protective immunity to cyclically transmitted *T. congolense* in cattle (Akol and Murray, 1985) and rabbits (Luckins *et al.*, 1983) is effective against the metacyclic population at the level of the skin as no detectable skin reactions develop at the site of bites by tsetse flies infected with homologous serodemes. The development of protective immunity to homologous challenge in cattle and rabbits coincides with appearance of metacyclic neutralizing antibodies in serum (Luckins *et al.*, 1983; Akol and Murray, 1985). This indicates that local expression of immunity might be due to presence of specific antibodies in the skin of the hosts.

The relative ease with which protective immunity against *T. congolense* and *T. brucei* can be induced, contrasts with the situation in *T. vivax*. Goats infected by tsetse transmitted *T. vivax* and then treated with a trypanocidal drug failed to develop comprehensive immunity to homologous challenge (De Gee *et al.*, 1980; Vos, Moloo and Gardiner, 1988b). In contrast, cattle which had recovered from infections induced by syringe inoculation of West or East African stocks of *T. vivax* were resistant to challenge by tsetse infected with homologous serodeme (Nantulya, Musoke and Moloo, 1986). However, it was not possible to demonstrate the presence of neutralizing antibodies to metacyclic forms of *T. vivax* in recovered animals at the time of challenge suggesting that the immunity induced was directed against bloodstream rather than to metacyclic VATs (M-VATs) (Nantulya *et al.*, 1986).

Only limited success has been achieved in attempting to protect goats against homologous challenge with *T. vivax* by infection with large numbers of culture-derived metacyclic trypanosomes followed by chemotherapy (Vos *et al.*, 1988b). Therefore, the role of metacyclic trypanosomes in induction of immunity in *T. vivax* is less clear than is the case with *T. brucei* and *T. congolense*. However, there are a number of possible reasons for the failure of induction of immunity to *T. vivax*. Tsetse infected with *T. vivax* transmit fewer metacyclics compared with either *T. congolense* or *T. brucei* (Otieno and Darji, 1979). In addition, *T. vivax* leaves the skin rapidly after infection, and induces only small and transient local skin reactions (Dwinger *et al.*, 1988) possibly resulting in poor antigenic stimulus against M-VATs.

2.5.4 Immunopathology

Among the pathological effects of the trypanosomiasis are extensive tissue lesions, progressive anaemia, structural changes in lymphoid organs, microvascular and circulatory changes and severe meningoencephalitis (Murray, 1979). These pathological changes have been attributed to trypanosome-derived biologically active substances, disturbances in hosts metabolic pathways or host factors derived from the excessive effects of inflammatory and immune responses to the parasite. Trypanosomes release a variety of biologically active products which include enzymes, lipids, haemolysins, mitogens and inflammatory factors (Tizard *et al.*, 1978b). These products have the potential to initiate a cascade of events capable of mediating tissue damage in infected hosts (Tizard *et al.*, 1978b). This hypothesis may be true for infections in laboratory rodents which develop acute infections characterized by high levels of parasitaemia. However, it is doubtful whether these parasite derived substances play a major role in pathogenesis of chronic infections developing in domestic animals and man which are characterized by low levels of parasitaemia. It is therefore likely that other mechanisms are operative in mediating tissue damage.

Extensive destruction of trypanosomes by antibodies *in vivo* results in generation of soluble antigen antibody complexes (Galvao-Castro, Hochmann and Lambert, 1978; Whittle, Mohamed and Greenwood, 1980; Mansfield, 1981). Immune complexes and host cellular responses might play a major role in the genesis of some of the pathological lesions observed in trypanosome infected animals. Such lesions do not develop in T cell deficient mice infected with trypanosomes unless they are reconstituted with syngeneic T cells, indicating that most of the lesions are mediated by the presence of T cells (Galvao-Castro *et al.*, 1978; Poltera *et al.*, 1980a,b).

Activation of the Kallikrein-Kinin system by immune complexes leads to release of pharmacologically active peptides such as histamine and 5-hydroxytryptamine (5-HT). Histamine and 5-HT increase vascular permeability causing extensive tissue oedema. Serum levels of histamine increase in *T. brucei* infected mice (Richards, 1965) and levels of 5-HT increase in *T. vivax* infected goats (Veenendaal and Van Miert, 1976). Extensive leucocytic infiltration into various tissues of infected host, is also thought to be due to release of chemotactic C4a and C5a through immune-complex mediated complement activation (Morrison *et al.*, 1985).

A major consequence of trypanosome infections is the depression of the host's immune system. During infection, the host responds to numerous emergent VATs but is less responsive to non-trypanosomal antigens and more susceptible to secondary infections. This is well documented in laboratory animals (Goodwin *et al.*, 1972; Murray *et al.*, 1974). The response *in vitro* to sheep red blood cells and the non-specific mitogen, lipopolysaccharide of splenic B lymphocyte from trypanosome infected mice is depressed (Corsini *et al.*, 1977; Roelants *et al.*, 1979; Sacks *et al.*, 1980). Cell mediated responses and T cell functions are also depressed. Splenic T lymphocytes from *T. brucei*, *T. congolense* or *T. equiperdum* infected mice show reduced *in vitro* proliferative responses in mixed leucocyte reactions (MLC) and to concanavalin A and phytohaemagglutinin (Corsini *et al.*, 1977; Moulton and

Coleman, 1977; Jayawardena and Waksman, 1977). Mitogen induced production of interleukin-2 (IL-2) and expression of IL-2 receptors (IL-2R) by T cells from *T. brucei* and *T. congolense* infected mice *in vitro* is also diminished (Mitchell, Pearson and Gaulde, 1986; Sileghem, Hamers and De Baetselier, 1987). *In vivo, T. brucei* infected mice show reduced delayed type hypersensitivity (DTH) reactions to oxazolone and dinitrofluorobenzene (Murray *et al.*, 1974b; Askonas *et al.*, 1979).

Several hypotheses have been proposed to account for the immunosuppression seen in laboratory rodents. This has been attributed to generation of suppressor, thymus derived lymphocytes (Jayawardena and Waksman, 1977; Eardley and Jayawardena, 1977; Wellhausen and Mansfield, 1979) or macrophages (Mansfield and Bagasra, 1978; Grosskinsky and Askonas, 1981), polyclonal stimulation and clonal exhaustion of bone marrow-derived lymphocytes (Freeman *et al.*, 1973; Hudson *et al.*, 1976), trypanosome derived mitogenic factors (Askonas *et al.*, 1979; Clayton *et al.*, 1979) or suppression of IL-2 production and IL-2 R expression by T cells (Mitchell *et al.*, 1986; Sileghem *et al.*, 1987). The degree of immunosuppression observed in large domestic animals is less severe than that seen in small laboratory animals. However, several studies have reported significant suppression of antibody responses to various bacterial and viral antigens in *T. congolense* infected cattle, sheep and goats (Griffin, Waghela and Allonby, 1980; Sharpe *et al.*, 1982; Rurangirwa *et al.*, 1983; Ilemobade *et al.*, 1982; Malu and Tabel, 1986; Mwangi, Munyua and Nyaga, 1990). Some results suggest that the lowered antibody responses are still able to confer sufficient protection (Sharpe *et al.*, 1982), while others report of impaired protective immunity (Rurangirwa *et al.*, 1979; Ilemobade *et al.*, 1982). The mechanisms which might be involved in immunosuppression in domestic animals infected with trypanosomes are more obscure. It is possible that some of the mechanisms suggested for small laboratory animals might be operative. The emergence of numerous VATs during the course of infection offers antigenic competition for immunocompetent cells with heterolgous antigens (Nantulya *et al.*,

1982) indicating that there might be defects in antigen handling and presentation by macrophages and other antigen presenting cells.

Sequential challenge of cattle with two unrelated serodemes of *T. congolense* results in the interference in establishment of the second infection by that already present (Morrison *et al.*, 1985). Similar observations have also been made in rabbits (Luckins and Gray, 1983) and goats (Dwinger *et al.*, 1989), when they were challenged 12 to 20 days after primary infection. Secondary challenge with an heterologous serodeme does not result in chancre development although low levels of neutralizing antibodies may be elicited. However, when Akol and Murray (1985) superinfected cattle at 95 days with unrelated serodemes of *T. congolense*, chancres developed and an increase in parasitaemia was noticed. Interference in establishment of secondary infection has also been observed between different trypanosome species (Dwinger *et al.*, 1990). Specific immunity does not appear to be the cause of interference and a requirement of an active infection is necessary since treatment of infected animals with berenil prior to challenge renders them fully responsive to secondary trypanosome infection (Morrison *et al.*, 1985). The mechanism involved in this phenomenon are not understood. It is possible that during infection, factors are produced which inhibit trypanosome growth or suppress production of substances required to promote growth of the second trypanosome serodeme, while the existing population has been selected for higher or lower avidity for such modulatory substances (Dwinger *et al.*, 1989).

2.6 The Local Skin Reaction (Chancre)

In African trypanosomiases, the early events that occur following establishment of trypanosomes in the skin play an important role in the development of initial immune responses, susceptibility of the host and pathogenesis of the disease. Following an infective tsetse fly bite and deposition of metacyclic trypanosomes into the skin of a susceptible host, a localized, indurated, erythematous skin reaction termed 'chancre' develops at the bite site four to 12 days later (Roberts, Gray and

Gray, 1969; Gray and Luckins, 1980; Akol and Murray, 1982). This local skin reaction is induced by an extravascular focus of trypanosomes undergoing proliferation and differentiation before entering the general circulation (Luckins and Gray, 1979).

The development of local skin reactions in cattle after tsetse fly bites had long been known to occur (Livingstone, 1857) but it was not until tsetse flies were proven to be vectors of pathogenic trypanosomes (Kleine, 1909) that these lesions were recognized as the first symptoms and an important aspect of human trypanosomiasis (Graf, 1929; Burtt and Fairbairn, 1945; Fairbairn and Godfrey, 1957). However, even then, little attention was paid to the occurrence of skin reactions in domestic livestock. The first experimental studies on skin reactions in cattle following bites of tsetse flies infected with *T. congolense* were made 100 years after the original reports by Livingstone (Bolton, 1965; Roberts *et al.*, 1969). Thereafter a series of experiments have been carried out in rabbits, cattle, sheep and goats as the skin reaction was recognized not only as the first clinical indication of the disease but also a vital stage of induction of immune response to trypanosomes.

In cattle, skin reactions develop five to 11 days after *T. congolense* infected tsetse fly bite and at least four days before parasitaemia is detected either microscopically or by inoculation into rats (Bolton, 1965; Roberts *et al.*, 1969). Similar reactions have been described in sheep and goats following infection with tsetse transmitted *T. congolense*, *T. brucei* or *T. vivax* (Uilenberg *et al.*, 1973; Gray and Luckins, 1980; Emery and Moloo, 1980, 1981; Dwinger *et al.*, 1987, 1988). Local skin reactions do not develop in mice, guinea pigs or rats probably due to the fact that they have a relatively thin skin and therefore the tsetse fly deposits trypanosomes deep into the musculature (Emery and Moloo, 1980). Local skin reactions show a similar course of development in hosts infected with different species of trypanosomes, varying only on the time of onset, size and duration of the lesions. In cattle, *T. congolense* induces development of hard raised clearly defined

palpable nodules measuring 10 to 20 mm in diameter five to seven days after infective tsetse bites (Roberts *et al.*, 1969; Akol and Murray, 1982). The nodules develop further into softer skin reactions with no distinct borders three to four days later. During this period they measure 15 to 30 mm in diameter (Roberts *et al.*, 1969; Akol and Murray, 1982). These nodules develop further into softer skin reactions with no distinct borders three to four days later. During this period they measure 15 to 30 mm in diameter (Roberts *et al.*, 1969). The reactions reach a maximum of 40 to 100 mm in diameter 10 to 13 days after infection when they appear as circumscribed indurated swellings which are hot and painful on palpation. On unpigmented skin these lesions are seen as swollen, red inflammed areas with grey centres (Akol and Murray, 1982). From 14 to 15 days, the reactions subside to form distinct plaques and by 20 to 30 days after infection they are indistinguishable from normal skin. A similar course of development is observed in rabbits, sheep and goats infected with *T. brucei* and *T. congolense* (Gray and Luckins, 1980; Uilenberg *et al.*, 1973; Emery and Moloo, 1980, 1981; Dwinger *et al.*, 1989). Skin reactions elicited by *T. vivax* are less spectacular as only small nodules are detected six days after challenge and only develop 12 to 24 hours before detection of trypanosomes in blood (Emery and Moloo, 1981).

Cattle become infected with *T. congolense* following intradermal inoculation of either bloodstream forms or small numbers (10^2 to 10^3) of metacyclic forms derived from tsetse flies. However only metacyclic forms induce local skin reactions (Akol and Murray, 1982). Similarly, metacyclic forms of *T. congolense* propagated *in vitro* (Gray *et al.*, 1981; Hirumi, Hirumi and Moloo, 1982) cause infection and induce skin reactions at the site of intradermal inoculation in rabbits, cattle and goats (Luckins *et al.*, 1981; Akol and Murray, 1986b; Dwinger *et al.*, 1987). The onset, size and duration of such lesions are dependent on the number of trypanosomes inoculated (Dwinger *et al.*, 1987).

2.7 Lymphocyte Migration and Localization in Sheep

Immunological research in trypanosomiasis has been carried out extensively in laboratory rodents and a considerable amount of information on host-parasite relationships has been obtained. However, rodents are unsuitable for studies involving metacyclic trypanosomes where establishment of an extravascular focus of trypanosomes has important consequences on the eventual outcome of infection. Although cattle would have been ideal hosts for the investigations reported on this work, constraints on animal accommodation, surgical manipulation and cost prevented their use. As an alternative host, sheep have several advantages. They are a natural host species, they are easy to accommodate and maintain, are amenable to a variety of surgical manipulation and more importantly the early stages of development of *T. congolense* in the skin are similar to those occurring in the bovine host. In addition, there is a wide body of knowledge on immune and inflammatory responses to introduced antigens or pyrogens, migration of lymphocyte populations between the vascular and lymphoid compartments and the modulatory effects of antigens upon these migratory pathways (Hay and Cahill, 1981). The surgical procedures which permit the insertion of cannulae into the afferent and efferent lymphatic ducts of lymph nodes have enabled the study of cell kinetics in the immune response to percutaneous or intralymphatic infusions of immunogens (Hall and Morris, 1965; Hay, Cahill and Traka, 1974) and in response to grafted tissues and organs (Hall, 1967; Pedersen and Morris, 1970). By use of such techniques the kinetics of entry of blood borne lymphocytes into lymphoid and other tissues may be monitored and the cellular and humoral products of immune reactions taking place within lymphoid and non-lymphoid tissues can be quantitatively and qualitatively assessed from the lymph issuing from such tissues.

The recent advent of hydridoma technology and the development of monoclonal antibodies to surface markers of sheep lymphocytes and other leucocytes has now permitted a more precise definition of the qualitative and quantitative cellular

kinetics of lymphocyte subpopulations in the immune response and their functional roles in immunity and pathogenesis of diseases (Mackay *et al.*, 1985; Puri, Mackay and Brandon, 1985; Hopkins *et al.*, 1986; Mackay *et al.*, 1986, 1987).

Lymphocyte and macrophage populations in sheep continuously migrate through tissues. Lymphocytes are carried passively in the blood and lymph, and they move in and out of the circulation tissues by virtue of their inherent mobility (Abernethy and Hay, 1988). Lymphoid cell migration is crucial to successful immune defence and the continued recirculation of small lymphocytes maximizes the opportunity of antigen presenting cells, effector cell precursors and regulatory cells of appropriate specificity to cooperate in response to antigen. The subsequent migration of effector cells to target sites ensures an appropriate dissemination of the immune response. The migration of cells through the skin, draining lymph nodes and peripheral blood is relevant to the early pathogenesis and immune responses in trypanosomiasis and in understanding the response to infection with metacyclic trypanosomes.

2.7.1 Migration of leucocytes through normal skin

The skin contains small numbers of lymphocytes and macrophages which migrate into the draining lymph node through the dermal lymphatic network. These lymphatics anastomose to form larger afferent collecting lymphatics. They have few open junctions, have one way valves and smooth muscles which by spontaneous contractions propel lymph towards the lymph node. Cells proceeding from the skin therefore can be collected by cannulating afferent lymphatic vessels (Hopkins *et al.*, 1985). It is possible to collect the peripheral lymph draining subcutaneous tissues in sheep over long periods - up to three months. Most of the cells collected are mononuclear cells in which lymphocytes predominate but also present are cells of the monocyte/macrophage lineage (Miller and Adams, 1977). The lymph also contains macrophage-like cells possessing cytoplasmic veils as described by Morris *et al.* (1972) which are in close association with lymphocytes. When peripheral lymph

nodes are surgically removed there is remarkable regeneration and rejoining of the lymphatic vessels to form a pseudoafferent duct, but the lymph node does not reform (Trevella and Morris, 1980). This makes feasible the long term collection of afferent lymph (Hopkins *et al.*, 1985).

2.7.2 Cell migration from lesions and areas of antigen deposition in the skin

Following injection of foreign material into the skin, alterations in content and composition of the peripheral lymph are observed (Hall and Morris, 1963; Hay and Cahill, 1982). The induction of chronic granulomatous lesions in drainage areas (Smith, McIntosh and Morris, 1970), grafting of tissues such as kidneys (Pedersen and Morris, 1970), injection of viable allogeneic lymphocytes (Hay and Cahill, 1982), injection of antigens to which a state of hypersensitivity exists due to appropriate previous exposure (Hay, Lachmann and Trnka, 1973) all cause an increase in the output of lymphocytes in peripheral lymph. The output of macrophage-like cells increases but the relative proportions of lymphocytes remains quite constant and similar to that seen in normal peripheral lymph.

2.7.3 Lymphocyte migration through lymph nodes

Lymph nodes are encapsulated secondary lymphoid tissues which are found throughout the body, superimposed on the lymphatic system. They act as filters for foreign materials which enter the peripheral lymphatics and provide a favourable environment for the interaction of different cell types in the generation of immune responses to lymph borne antigens. The structure of the node is in a highly dynamic state, with large numbers of lymphocytes continuously entering the node from the blood and afferent lymph and leaving by way of the efferent duct lymph (Morris, 1972). The lymph node is permeated by a system of sinuses. Multiple afferent lymphatics enter the lymph node capsule and empty into the subcapsular or marginal sinus. Lymph may then pass via cortical or intermediate sinus into an anastomosing system of medullary sinuses of the node. These sinuses then converge at the hilus where they are continuous with one or more efferent lymphatic vessels. The cortex

of the lymph node contains densely packed lymphocytes with B cells organized into distinct, spherical follicles and T cells present in the interfollicular areas. It is within the paracortical areas that lymphocytes enter the lymph node from the blood through specialized post-capillary venules (Gowans and Knight, 1964). Some of the lymphoid tissues extend into the medulla and are supported by strands of connective tissue fibres. These medullary cords contain a majority of plasma cells in addition to numerous phagocytic cells.

As lymph passes through the node, antigens are removed by phagocytic cells lining the medullary sinuses and also become available to cortical macrophages and lymphocytes for processing. Cells entering the node from either the lymph or blood localize preferentially in distinct areas of the node. Lymphocytes entering the node from the blood appear first in the follicular areas in the paracortex and then migrate into the cortical and medullary sinuses eventually leaving the node by way of the efferent lymph (Howard, Hunt and Gowans, 1972). Some of the migrant cells appear in the efferent lymph from lymph nodes within an hour or so of their injection intravenously but many are retained in the nodes for much longer (Fahy *et al.*, 1980). It has been suggested that in lymph nodes, migrant cells from the blood have access to only a restricted part of the node. So-called traffic areas have been designated in lymph nodes (Parrott, Sousa and East, 1966) and structures such as germinal centres have been shown to lie outside the migratory path of cells entering lymph nodes from the blood (Parrott, 1967; Austin, 1968; Howard *et al.*, 1972).

Only about 30% of unstimulated lymphocytes labelled with ^{3}H-thymidine infused into a lymph node by way of afferent lymph leave the node in the efferent lymph during 48 hour periods following infusion. The cells which remain in the node migrate rapidly out of the subcapsular and cortical sinuses and become distributed throughout the superficial and deep cortex within a short time of entering the node (Fahy *et al.*, 1980).

2.7.4 Cell migration and localization in antigen stimulated lymph node

Following antigen stimulation of the skin, lymphoproliferation and increased recruitment of cells from blood and lymph occurs causing the gross enlargement of the lymph node. T-dependent antigens cause active T cell proliferation in the paracortical areas while T-independent antigens result in proliferation of B lymphocytes in the cortex. The response normally observed is a combination of the two; namely a generalized hyperplasia due to heterogen eity of various antigens. Germinal centres and secondary follicles are seen containing actively proliferating B cells. Secondary follicles contain dendritic cells, some macrophages and few T cells and natural killer cells. Together with the specialized macrophages of the marginal sinus these cells play a role in B cell response and development of B-memory cells.

2.7.5 Cells in efferent lymph

The traffic of cells into efferent lymph is well documented. Lymphocytes are the predominant cellular constituents since macrophages are not normally found in the efferent lymph. About 3×10^7 lymphocytes leave the node via efferent lymph every hour. Only 10% of efferent cells come from the afferent lymph ducts. Local irradiation of a single sheep lymph node does not result in obliteration of cellular output (Hall and Morris, 1964). The majority of efferent lymphocytes are not generated within the lymph node but are derived from blood perfusing the node. The formal proof of this was obtained by continuous infusion of ^{3}H-thymidine into a sheep popliteal lymph node through an indwelling catheter in an afferent lymphatic. Less than 4% of the cells found in efferent lymph could be labelled by ^{3}H-thymidine infusion throughout the whole course of the experiment indicating that the majority of efferent lymphocytes were not newly made in the node but were blood borne (Hall and Morris, 1965) at least in a normal lymph node.

Studies on the response of single lymph nodes to antigenic stimulation revealed that dramatic changes occur in the cell content of efferent lymph. In the first 24 hours after antigenic challenge cell traffic is greatly reduced. Subsequently, cells are recruited from the blood into the node, where they undergo a process of selection,

proliferation and differentiation into antibody synthesizing cells. At 72 to 96 hours after challenge the cell output in the lymph increases to a maximum of five to 10 times above prestimulation level and large blast cells and antibody-forming cells appear. The cells leaving the node disseminate the immune response systemically and widespread immunological memory is established (Fahy *et al.*, 1980).

CHAPTER THREE
GENERAL MATERIALS AND METHODS

3.1 Experimental Animals

Adult female and castrated male sheep of either Scottish Blackface, Suffolk or small Finnish Landrace breeds aged 12 to 18 months were used. These animals were tagged for identification, kept indoors in pens in the CTVM animal unit and were all drenched with 5 mg/kg bodyweight of fenbendazole (Panacur 2.5% Hoechst, Frankfurt, W. Germany). Two weeks prior to infection, they were transferred to a flyproof, air-conditioned urban isolation unit with concrete pens on a floor covered with wood shavings. The sheep had free access to hay and water with limited supply of concentrates.

3.2 Trypanosomes

Three stocks of *T. congolense*, TREU 1457, TREU 1881 and TREU 1885, were used in this study. *T. congolense* TREU 1457 is a cloned derivative of TREU 1290 and was originally isolated from an ox in Zaria, Nigeria in 1967 as Zaria/67/LUMP/69 (Luckins and Gray, 1983). *T. congolense* TREU 1881 (primary isolation code DA/ZM/81/TRPZ/105) and TREU 1885 (primary isolation code DA/ZM/81/TRPZ/132) are cloned derivatives of TREU 1842 and TREU 1851 respectively (Frame, Ross and Luckins, 1990). They were originally isolated from dogs in Kakumbi, Chipata District of Zambia. A detailed history of the trypanosome stocks used is given in Appendix I.

3.2.1 *In vitro* cultivation

Cultures containing infective metacyclic forms of *T. congolense* were maintained and passaged as described by Gray *et al.* (1984, 1985) and were kindly supplied by Dr. Carole Ross and colleagues. Cultures were gassed with 5% CO_2 in air and incubated at 28° in Falcon Primaria T-25 flasks in medium prepared from a powdered form of Eagles Minimum Essential Medium (EMEM) and supplemented with 4 mM glutamine, 20 mM HEPES buffer, 2.2 g/l sodium bicarbonate and 20% v/v foetal bovine serum which was heat inactivated at 56°C for 30 minutes before use. Cultures consisted of dividing epimastigote trypanosomes in addition to infective non-dividing metacyclic trypanosomes (Gray *et al.*, 1984).

3.2.2 Separation of metacyclic forms

To separate metacyclic forms of *T. congolense* from epimastigote forms, approximately 15 mls of freshly harvested culture supernatant was placed on a column of diethylaminoethyl cellulose, DE-52 (Whatman Lab. Sales) (Gray *et al.*, 1984) equilibrated with phosphate buffered saline pH 8.0 containing 1% (w/v) glucose (PSG). The DE52 was packed in a 50 ml hypodermic syringe barrel (Gray *et al.*, 1984). The syringe barrel outlet was plugged with 5 mm of sterile glass wool. A filter paper disc (Whatman) was placed on the surface of the packed DE-52. Metacyclic forms were eluted with 60 mls of PSG, collected in sterile universal bottles on ice and then centrifuged at 2260 g for 20 minutes at 4°C. The packed organisms were resuspended in 1 ml of PSG and the number of metacyclics per ml in the total eluate obtained by counting on an improved Neubauer haemocytometer. The concentration was then adjusted and standardized to contain 1 x 10^6 metacyclic trypanosomes per ml of PSG. In some instances, when there were few metacyclics in culture supernatant, the contents of up to 10 flasks was pooled, centrifuged at 2260 g for 15 minutes at 4°C and the packed organisms resuspended in 15 mls of supernatant before passing through a column of DE-52 as described above. Procedures for preparing PSG and DE-52 are given in Appendix II.

3.3 Determination of Clinical and Parasitological Parameters

3.3.1 Rectal temperatures

Daily rectal temperatures were monitored using a digital thermometer every morning at 9.00 a.m.

3.3.2 Packed cell volume

Two ml amounts of blood were collected from the jugular vein into tubes containing sodium ethylenediamine tetra-acteic acid (EDTA). Packed red cell volume percent (PCV) was determined by a microhaematocrit method. Blood was drawn into capillary tubes up to two thirds full and heat-sealed at one end. The tubes were then labelled and placed in a microhaematocrit centrifuge (Biofuge, Heraeus Sepatech) and

centrifuged at 12000 g for five minutes. The PCV was determined using a microhaematocrit reader (Hawksley).

3.3.3 Parasitaemia/parasitosis

Glass capillaries were filled with jugular blood in EDTA, heat-sealed and centrifuged at 12000 g for five minutes in the microhaematocrit centrifuge. The leucocyte/plasma interface was examined for trypanosomes by direct examination of the capillary tubes under phase contrast microscopy using a x10 objective (Woo, 1970). This technique was combined with examination of unstained wet blood films. These were prepared by placing a drop of blood obtained from the ear vein of the sheep onto a clean slide and then overlaid with a coverslip. Up to 50 microscope fields were searched for trypanosomes using phase contrast microscopy and a x40 objective. The estimated parasitaemia was then expressed as numbers of trypanosomes/field. In lymph, trypanosomes could usually be observed using fresh wet films, but when the number of trypanosomes was low, the lymph was concentrated 10 times by centrifuging 1 ml of lymph at 12000 g for five minutes and then resuspending the pellet in 0.1 ml of the supernatant. Trypanosomes were counted and parasitosis was expressed as $\log_{10}$ of trypanosomes per ml of lymph.

3.4 Preparation of Peripheral Blood Leucocytes

Two methods of preparation of peripheral blood leucocytes were employed. The first technique, density gradient centrifugation of blood to separate mononuclear cells (MNCs) from red cells and granulocytes, was performed initially in order to obtain relatively pure MNCs which could be analyzed by fluorescence microscopy. In later experiments, peripheral blood leucocytes were prepared by ammonium chloride lysis technique and cells analyzed by flow cytometry. Granulocytes were gated out during cell analysis.

3.4.1 Preparation of peripheral blood mononuclear cells by density gradient centrifugation

Blood was collected from the jugular vein of sheep into sterile glass universal bottles and defibrinated using sterile glass beads. Defibrinated blood was then

transferred into clean plastic universal containers and centrifuged at 1000 g for 20 minutes at 15°C. The buffy coat layer, approximately 5 to 10 ml, was carefully aspirated off and thoroughly mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2, by gentle inversion, then layered on top of 8 ml Ficoll/Sodium Metrizoate ('Lymphoprep' specific gravity 1.076, Nycomed AS, Diagnostica, Torshov, Norway). Cells were centrifuged at 1000 g for 30 minutes at room temperature (Mackay *et al.*, 1985). The mononuclear cells at the plasma gradient interface were recovered using a pasteur pipette. Cells were then washed three times in Hanks balanced salt solution (HBSS) pH 7.2 containing 1% bovine serum albumin (BSA) by centrifugation at 350 g for 10 minutes at 4°C. After the final wash, they were resuspended in 5 mls of cold HBSS-BSA, and the concentration determined by counting viable cells in a Neubauer haemocytometer. The viability of cells was determined by trypan blue dye exclusion test (Hudson and Hay, 1989). Cell concentration was adjusted to 10^7/ml in HBSS-BSA and the suspension kept at 4°C.

3.4.2 Preparation of peripheral blood leucocytes by Tris-ammonium chloride lysis (Mishell and Shigi, 1980)

Ten ml of jugular blood was collected from sheep into vacutainers containing lithium heparin (Becton-Dickinson). Five ml amounts of blood were mixed with 17.5 mls of prewarmed (37°C) Tris-ammonium chloride solution (Appendix II). All red cells were lysed within three minutes. The lysed blood was then centrifuged at 800 g for 15 minutes at 15°C. The pellet containing leucocytes was resuspended in PBS pH 7.2 containing 1% BSA and 0.1% sodium azide (Immunofluorescence buffer, IMFB) and then washed twice by centrifugation. Cells were counted, the percentage of viable cells determined and the concentration adjusted to 10^7 per ml in IMFB.

3.4.3 Preparation of cytocentrifuge smears

Cytocentrifuge smears were prepared to determine differential leucocyte counts in cell suspensions or lymph samples. A cytocentrifuge (Shandon Southern Instruments Ltd., Cheshire, UK) was loaded with block holders containing sample chambers, clean labelled glass slides and filter cards. The cell suspension was

adjusted to 1 x 10^5 cells/ml and 50 μl of cell suspension dispensed into the sample chamber and centrifuged at 800 g for five minutes. After centrifugation, slides were air dried, and fixed in 95% methanol (BDH) for one minute. To examine the morphology of cells, cytocentrifuge smears were stained in 5% Giemsa's stain in Giemsa buffer (Gurr, BDH) for 40 minutes. They were then rinsed in the same buffer, air dried and examined by light microscopy. Differential leucocyte counts were made by counting at least 200 cells under oil immersion using a x100 objective.

3.5 Collection and Sampling of Skin Biopsies and Lymph Nodes

Skin biopsies were obtained from infection sites under local anaesthesia (Dwinger *et al.*, 1988). The area around the infection site was shaved, cleaned and infiltrated subcutaneously into several sites with 5 ml of 2% Xylocaine (Astra Chemicals, Penelux, BV, Rijswijk, Holland) in the form of an "L"-block. An elliptical piece of skin measuring approximately 3 cm in length including the centre of the infection site and encompassing normal skin at each extremity, together with epidermis, dermis and panniculus carnosus (cutaneous) muscle was excised using a scapel blade. The wound was closed using single mattress sutures (Nylon $^2/_0$, Ethicon) and tetracycline spray was applied to the wound.

Lymph nodes draining local skin reactions were obtained after sheep were killed by intravenous injection of 15 mls of sodium pentobarbitone (Euthatal, May and Baker Ltd., Dagenham, England) followed by exsanguination. Skin and lymph node samples were divided into two. One half of each sample was placed in either neutral buffered formalin or Bouin's solution for conventional histology. The other half was snap-frozen in dry ice and isopentane wrapped in aluminium foil, sealed in plastic bags and then stored at -70°C until used for immunohistology. Lymphocytes and other cellular phenotypes were localized in cryostat sections of frozen tissues by indirect immunoperoxidase technique (Barclay, 1981; Meeusen *et al.*, 1989).

3.6 Pseudoafferent and Efferent Lymphatic Duct Cannulation

3.6.1 Preparation of pseudoafferent lymphatic ducts

Since afferent lymphatic ducts draining the skin are small and delicate they are difficult to cannulate. However, surgical removal of the draining lymph node results in anastomoses of the afferent ducts with efferent(s) of the removed node to form 'pseudoafferent' ducts six weeks later. The lymph node does not regenerate and the pseudoafferent lymphatic duct is easier to cannulate since it is larger.

The prefemoral lymph node in sheep is located superficially on the cranial border of the *tensor fasciae latae* muscle, between the tuber coxae and the patella. It is laterally bound by the deep surface of the cutaneous muscle (*panniculus carnosus*). The lymph node receives afferent lymphatics from the skin of the pelvic and cranial part of the thigh areas (Sissons and Grosman, 1985), (Figure 3.1). The duct(s) accompany the circumflex iliac blood vessels on the deep surface of *tensor fasciae latae* and then enter the abdominal cavity where it connects with either lateral or iliac lymph nodes or join the lumbar lymphatics ducts directly (Figure 3.2).

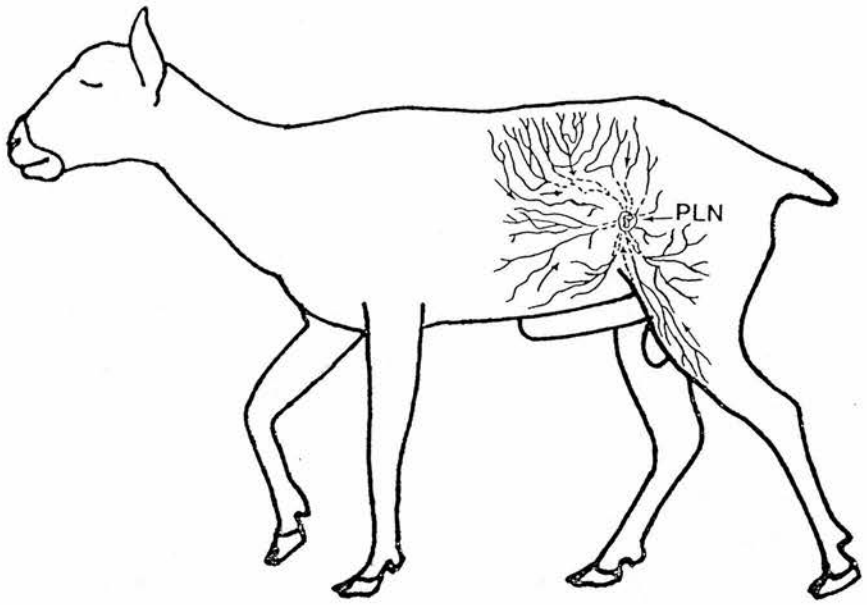
Prior to surgical removal of prefemoral lymph nodes, sheep were starved for 18 hours. The flanks and the neck regions were sheared closely. Anaesthesia was induced by intravenous injection of alphaxalone/alphadolone (SAFFAN, anaethestic injection, Glaxovet Ltd., Uxbridge, Middlesex, England) into the jugular vein. Sheep were intubated using a cuffed endotracheal tube (size 9). Halothane (Halothane B.P., RMB Animal Health Ltd., Dagenham, England) was administered through the endotracheal tube connected to a closed circuit anaesthetic machine for maintenance of anaesthesia. Initially, 8% halothane was administered and then after approximately five minutes reduced to a constant 4% with an oxygen flow rate of 0.2 l/min. and 0.2 l/min. nitrous oxide during the operation.

The surgical area was disinfected using 0.5% chlorhexidine gluconate B.P. (Savlon, hospital concentrate, ICI, Macclesfield, Great Britain) and 70% alcohol. The lymph node was identified by palpation and a 6 cm long skin incision made parallel

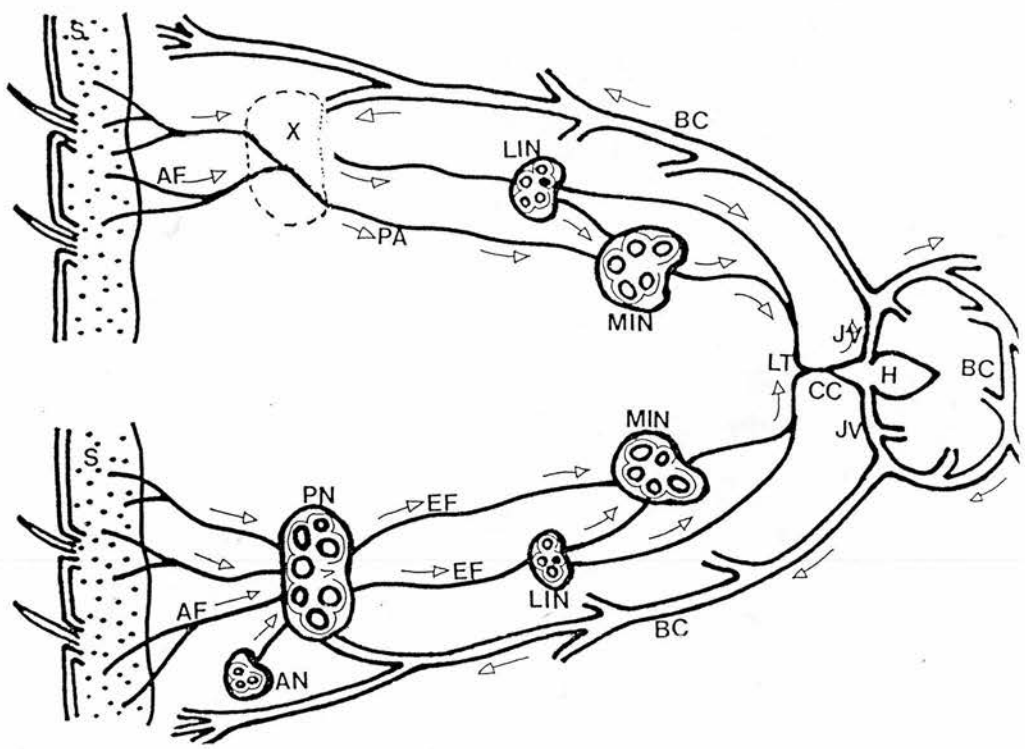
FIGURE 3.1 Diagram showing the areas drained by afferent lymphatics of prefemoral lymph node (adapted from Sisson and Grossman's. The Anatomy of the Domestic Animals, Vol. 1, Editors R. Getty, W.B. Saunders Company, Philadelphia, London, Toronto, 1975).

FIGURE 3.2 Schematic diagram showing lymph flow from areas drained by prefemoral node in sheep and indicating the reanastomosis of afferent lymphatics after lymphadenectomy. S, Skin; AF, Afferent lymphatic duct; AN, Accessory lymph node; PN, Prefemoral lymph node; X, Position of ablated lymph node; EF, Efferent lymphatic duct; PA, Pseudo-afferent lymphatic duct; LIN, Lateral iliac lymph node; MIN, Medial iliac lymph node, LT, Lymphatic trunk; CC, Cysterna chyli; H, Heart; JV, Jugular vein; BC Blood Circulation (adapted from Sissons, Grossman, 1975 and Akol, 1985).

3.1



3.2



and close to the lymph node. Bleeding was controlled by thermalcautery. The node was separated from the subcutaenous fat by blunt dissection and held using a pair of Allis tissue forceps. Blood vessels to the nodel were identified, ligated and divided before removal of the node. Any accessory nodes present were identified and removed. The wound as then closed using $^{2}/_{0}$ chromic catgut (Ethicon Ltd., Edinburgh, Scotland) in a continuous suture for the subcutaneous tissue and cutaneous muscle. The skin was closed using Michel clips. The operation was repeated for the opposite or contralateral prefemoral node. Sheep were then housed in a clean well bedded pen for eight weeks.

3.6.2 Lymphatic duct cannulation

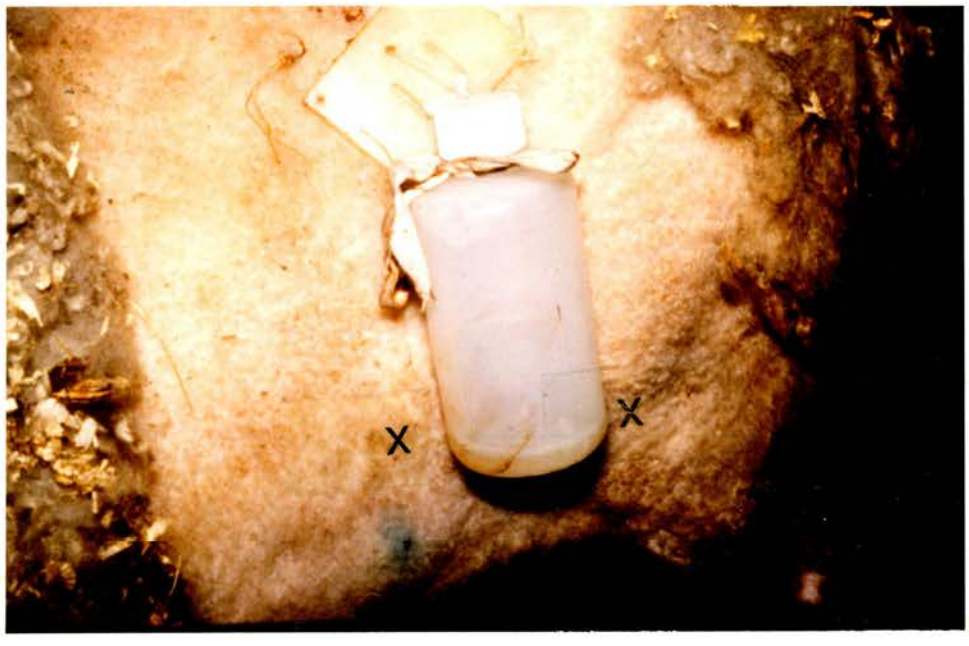
Surgical cannulation of pseudoafferent lymphatic duct was performed according to techniques described for prefemoral efferent lymphatic duct by Hall (1967). Induction and maintenance of anaesthesia and preparation of the surgical site was as described in Section 3.6.1. A sterile solution of Evans blue dye was injected intradermally into the draining area for easy delineation of the lymphatic duct. With the animal in lateral recumbency, the surgical approach was begun by making a 10 cm long longitudinal incision through the skin and cutaneous muscle starting just below the region of the tuber coxae and following the anterior border of the thigh. By blunt dissection of the underlying subcutaneous fat, the inner surface of tensor fascia latae was exposed and retracted upwards and backwards using self retaining retractors to have a view of the deep surface. The circumflex iliac blood vessels were clearly visible associated with the pseudoafferent duct and the nerve running in a dorso-posterior direction. If several accessory ducts were present, the largest and most accessible duct was selected for cannulation and ligated ($^{2}/_{10}$ Silk, Surgisilk, Sutures Ltd., U.K.). If present, smaller lymphatics were ligated and divided. The duct was then freed from blood vessels, nerve and adhering connective tissue. Two other sutures were placed loosely around the cleared duct. A sterile polyvinyl cannula (Portex) with an internal diameter of 0.58 mm and external diameter of 0.95 mm was

led into the superficial site through a stab incision near the origin of *tensor fascia lata.* The cannula was sterilized by swabbing with 70% alcohol before being positioned in the direction of the lymphatic duct. The cannula was then flushed with heparin (Sigma) which was contained by clamping one end with an artery forceps. A transverse incision less than half the circumference was cut in the wall of the duct using scissors. The free end of the cannula was then inserted into the duct through the incision for a distance of 1 cm against the flow of lymph. Ligatures were then tightened to secure the duct around the cannula. The clamp on the cannula was then released from the opposite end and the flow of lymph verified. Other ligatures placed around the duct were tightened to anchor the cannula to the surrounding tissues. After establishment of lymph flow, the wound was closed as described earlier (Section 3.6.1). The stab wound through which the loose end of the cannula emerges was tightened using a purse-string suture.

In order to secure the external part of the cannula and to facilitate lymph collection, a small sheet of polythene 5 mm thick and approximately 7 x 5 cm was sutured to the skin of the sheep near the point of exit of the cannula using linen (Surgilin $^{4}/_{0}$ Sutures Ltd., Clwyd, U.K.). Lymph was collected into sterile 250 ml polythene bottles. A hole, 2 mm in diameter was made into the polythene screw cap of the collecting bottle in order to receive the cannula. A piece of tape was passed round the neck of the bottle and secured to the polythene sheet (Figure 3.3). The sheep received 1 ml of heparin subcutaneously around the drainage area, and 5 mls of penicillin/streptomycin by intramuscular injection. The sheep were then kept in metabolism cages for the period of the experiments. The procedure for prefemoral efferent lymphatic duct cannulation on intact sheep was similar to that described for pseudoafferent lymphatic ducts.

Lymph was collected into bottles containing 1000 iu heparin and powdered penicillin and streptomycin. Daily collections were made every morning and the volume of lymph recorded and flow rate calculated. Cell counts were made using

FIGURE 3.3 The site of pseudoafferent lymphatic duct cannulation in sheep showing an attached polythene bottle to collect lymph. The sheep was infected in the marked areas (X). The area stained blue (arrow) indicates the point of injection of a marker dye to delineate the lymphatic duct for ease of identification.


X
X

coulter counter and cytocentrifuge smears were prepared for determination of differential leucocyte counts. Cell counts and flow rates were used to calculate hourly cell output. All afferent and efferent lymph samples were collected daily from the day of infection.

3.7 Monoclonal Antibodies Specific for Ovine Leucocyte Subsets

Nine monoclonal antibodies (MAbs) used in this study were purchased from Sheep Biology Unit (SBU), Department of Veterinary Preclinical Sciences, University of Melbourne, Australia. They were supplied as ascites or tissue culture supernatants. Three other MAbs, SW73.2, VPM32 and VPM33 were obtained from the Department of Veterinary Pathology, University of Edinburgh as tissue culture supernates. The reactivity of the MAbs with sheep leucocyte antigen is shown in Table 3.1. In most cases, these antibodies identify antigens with analogues in other species and wherever appropriate the cluster of differentiation or CD nomenclature used to classify human lymphocyte antigens has been adopted (Bernard *et al.*, 1984).

3.7.1 Sheep leucocyte antigens identified by specific monoclonal antibodies

Sheep CD5 (SBU-T1)

This antigen is identified by MAb 25-91. The CD5 molecule in man is commonly referred to as T1 or Leu-1, in mouse Ly-1 and in rat MRC OX-19. In all three species, the CD5 molecule consists of a single polypeptide chain of 67 Kd and the same molecular weight (MW) value has also been found for sheep CD5 (Mackay *et al.*, 1985). The functions of CD5 in sheep and other species has not been determined but, in all species studied CD5-specific MAb augments the proliferation of T cells *in vitro* in response to various mitogens and alloantigens (Dallman *et al.*, 1984). The tissue distribution of sheep CD5 appears to be identical to that reported for CD5 in other species, with all thymocytes and most T cells displaying this molecule. Medullary thymocytes express higher levels of CD5 than the majority of cortical thymocytes (Mackay *et al.*, 1985). In sheep 20 to 50% of B cells express low

TABLE 3.1 Characteristics and reactivity of monoclonal antibodies with sheep leucocytes

Antigen specificity of monoclonal antibodies	Isotype	Sheep molecule	Cells identified
SBU-T1	IgG_1	CD5	Most T cells and a subpopulation of B cells
SBU-T4	IgG_{2a}} IgG_1 }	CD4	MHC Class II restricted T cells
SBU-T8	IgG_{2a}	CD8	MHC Class I restricted T cells
SBU-T19	IgG_1	T-19	$\gamma\delta$ T cells
SBU-I	IgG_1	MHC Class I	All somatic cells
SBU-T6	IgG_1	(CD1)	Cortical thymocytes, dendritic cells, and some B cells in peripheral blood
SBU-II	IgG_1	Limited part of MHC Class II	B cells, activated T cells, some macrophages, epithelial & dendritic cells
SBU-LCA	IgG_{2a}	CD45	All leucocytes
SBU-LCA p220	IgG_1	CD45R	B cells, some T cells
SW73.2	IgG_{2a}	Total MHC Class II	B cells, activated T cells. Macrophages/ monocytes dendritic cells and epithelial cells.
VPM32	-		All macrophages
VPM33	-		Macrophages and B cells

Abbreviations

CD - Cluster of differentiation
IgG - Immunoglobulin G
LCA - Leucocyte common antigen
MAb - Monoclonal antibody
MHC - Major histocompability complex
SBU - Sheep biology unit
SIg^+ - Surface immunoglobulin positive
VPM - Veterinary pathology monoclonal
$\gamma\delta$ - gamma/delta

levels of CD5. SBU-T1 has been used as a 'pan T cell' marker in sheep (Mackay *et al.*, 1988).

CD4 (SBU-T4)

This molecule is identified by two MAbs, 44.38 and 44.97 (Maddox *et al.*, 1985; Mackay *et al.*, 1986). The CD4 molecule in mammals is present on a subset of T cells which is MHC Class II restricted and chiefly responsible for helper/inducer functions (Reinherz and Schlossman, 1980; Mason *et al.*, 1983). CD4 has also been identified on some cytotoxic T cell subsets (Mackay, Maddox and Brandon, 1987). CD4 in mammals has a characteristic distribution being present on a majority of $CD8^-$ T cells, cortical thymocytes, most medullary thymocytes and cells of the macrophage and Langerhans cell lineage(s). This distribution holds true for sheep (Maddox *et al.*, 1985; Mackay *et al.*, 1986).

CD8 (SBU-T8)

CD8-specific MAbs of sheep (38.65), humans and rodents mark a population of mature lymphocytes which are $CD4^-$. $CD8^+$ cells, recognize antigens in association with MHC Class I molecules in humans and rodents and exhibit cytotoxic or suppressor functions (Reinherz and Schossman, 1980; Mason *et al.*, 1983). This association has not been determined in sheep but the molecular nature and tissue distribution of sheep CD8 resembles that of other species (Maddox *et al.*, 1985). In sheep, MAbs to CD8 stain 10 to 25% of peripheral lymphocytes as well as most cortical thymocytes and $CD4^-$ medullary thymocytes.

SBU-T19

SBU-T19 is an antigen on sheep lymphocytes identified by MAb 19.19 and is present on cells which are $CD4^-$, $CD8^-$, surface immunoglobulin (SIg) negative, but $CD5^+$ (Mackay *et al.*, 1986). These cells are now thought to represent gamma/delta T cells. In peripheral lymphoid organs, SBU-$T19^+$ cells are found almost exclusively in the small lymphocyte population and exhibit the physical characteristics of normal recirculating lymphocytes. This subset is responsive to T cell mitogen (Mackay *et al.*,

1986). The tissue distribution of SBU-T19$^+$ cells reveals a predominance in peripheral blood and afferent lymph, representing 10 to 30% of all lymphocytes, with relatively reduced numbers in the lymph node and efferent lymph. In lymph node sections, SBU-T19$^+$ cells are localized along subcapsular sinuses and trabeculae which extend from the capsule into the node (Mackay *et al.*, 1986).

CD45 (SBU-LCA) Leucocyte common antigen

MAb 1 to 28 defines an antigen which, based on tissue distribution and molecular weight (MW), is an analogue of the leucocyte common antigen (LCA) of rat, human and mouse (CD45). The CD45 molecule is essential for activation of protein tyrosine kinase during lymphocyte triggering. In sheep, this antigen is present only on cells of the haemopoietic lineage and is expressed on all lymphocytes of thymus, spleen, lymph nodes, ileal Peyer's patches (IPP) and peripheral blood, as well as being found on macrophages and granulocytes (Maddox *et al.*, 1985). SDS-PAGE analysis of LCA isolated from lymphocytes reveals MWs 190 Kd, 210 Kd and 225 Kd. However, different MWs of ovine LCA are found on different lymphocyte populations. B lymphocytes lack the 210 Kd form found on T lymphocytes and thymocytes while thymocytes lack the 225 Kd form found on B lymphocytes. In addition, one MAb, 20 to 96 has been produced which reacts with a component of LCA (CD45R) and immunoprecipitates a 220 Kd component of LCA (Mackay, Maddox and Brandon, 1987). This MAb reacts with all B cells, 'null' cells (CD5$^-$, CD4$^-$, CD8$^-$, SBU-T19$^-$, SIg$^-$) and some T cells, but reacts particularly well with peripheral B cells, giving rise to brightly stained cells by immunofluorescence.

Major histocompatability (MHC) antigens of sheep (ovine leucocyte antigens, OLA)

MHC Class I antigens have been characterized in sheep using MAb 41 to 19 which identifies all peripheral leucocytes and most somatic cells but only 20 to 40% of thymocytes (Gogolin-Ewens *et al.*, 1985). MHC Class I molecules are involved in recognition of antigen by cytotoxic T cells. MAb 28.1 and SW73.2 identify MHC Class II antigens. MHC Class II antigens are present on a majority of B cells, some

macrophages/monocytes, epithelial cells and activated T cells (Puri, Mackay and Brandon, 1985; Hopkins *et al.*, 1986). MHC Class II molecules are involved in recognition of antigen by $CD4^+$ cells (Mackay *et al.*, 1986).

CD1 (SBU-T6)

CD1 (SBU-T6) antigen in sheep is identified by MAb 20.27. Cells bearing this antigen include cortical thymocytes, langerhans cells, 'veiled' cells of afferent lymph and dendritic cells within the paracortical areas of lymph nodes (Mackay *et al.*, 1985). Tissue distribution of SBU-T6 is similar to the total human CD1 as it is detectable at low levels on peripheral blood B cells (Mackay *et al.*, 1985).

Fc receptors on macrophages and B cells

MAbs VPM32 and VPM33 identify antigens present on sheep macrophages and macrophages/B cells respectively.

3.8 Identification and Quantification of Cellular Phenotypes in Peripheral Blood and Lymph by Immunofluorescent Staining

3.8.1 Analysis of PBLs using fluorescence microscopy

3.8.1.1 Monoclonal antibodies and immunoconjugates

MAbs identifying CD5, CD4, CD8, MHC Class I and MHC Class II were used in the analysis of peripheral blood lymphocyte subpopulations. Although it was recommended that tissue culture supernatants of MAbs be used undiluted, it was found necessary to titrate and determine the optimal working dilutions which would give strong specific fluorescence and low background staining. MAbs were diluted two-fold from 1:2 to 1:8 and tested on sheep PBLs each with a range of immunoconjugate dilutions. The optimal dilutions were then made up in IMFB, stored at 4°C and used within five days.

Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin (GAM/Ig/FITC, Nordic Immunological Laboratories, Tilburg, Netherlands) was used as a second antibody for indirect immunofluorescence staining of cells. The lyophilized immunoconjugate was reconstituted in 1 ml of sterile distilled water, and stored at 20°C in 100 μl aliquots. The working dilution of the

immunoconjugate was determined by testing on sheep PBLs using several MAbs at their optimal dilutions. A 1:80 dilution of the FITC conjugate was optimal as it gave strong specific fluorescence and low background staining.

Surface immunoglobulin positive cells (SIg^+) were identified by direct immunofluorescence staining using FITC conjugated donkey anti-sheep/goat IgG (DAS/IgG-FITC; Scottish Antibody Production Unit, SAPU, Law Hospital, Carluke, Lanarkshire, Scotland). Two-fold dilutions from 1:10 to 1:80 were titrated with sheep PBLs and assessed for degree of fluoresence and non-specific staining. The optimal dilution was 1:40.

3.8.1.2 Procedure

The method used was essentially that described by Mackay *et al.* (1988). Sheep PBLs were adjusted to a concentration of 1 x 10^7 cells/ml. 50 μl of this suspension (5 x 10^5 cells) was then dispensed into wells of 'V' bottomed microtitre plates (Alpha Labs) and reacted with 50 μl of the appropriate MAb at 4°C for 30 minutes. Plates were then centrifuged at 350 g for five minutes at 10°C. The cells were then washed by centrifugation three times by gently removing the supernatant and then resuspending the pellet of cells with 150 μl of IMFB. After a final wash, the cell pellet was resuspended in 50 μl of 1:80 dilution of GAM-FITC conjugate and incubated at 4°C in the dark for 30 minutes. After staining, excess conjugate was removed by washing the cells three times in IMFB. Cells were then fixed in 150 μl of freshly made 1% paraformaldehyde in PBS.

For SIg^+ staining 5 x 10^5 cells in 50 μl of IMFB were dispensed into wells of 'V' bottomed microtitre plates, reacted with 50 μl of a 1:40 dilution of DAS-FITC and incubated for 30 minutes at 4°C. After washing twice in IMFB, the stained cells were fixed in 150 μl of 1% paraformaldehyde as described above.

3.8.1.3 Analysis and enumeration of cells

Twenty microlitres of the fixed, stained cell suspension was placed onto a clean glass slide and covered with a 22 x 22 mm coverslip. The edges were sealed

with nail varnish and cells observed under a fluorescence/phase contrast ultraviolet (UV) microscope with incident light (Leitz Laborlux K). For each field, fluorescent cells were counted under UV light (green fluorescence) while the total number of cells was determined using phase contrast microscopy. A total of more than 200 cells was counted for each sample. The number of fluorescent cells was then expressed as a percentage of the total number of cells.

3.8.2 Analysis using flow cytometry

3.8.2.1 Monoclonal antibodies and immunoconjugates

MAbs identifying CD5, CD4, CD8, SBU-T19, TCR, CD1, MHC Class I, MHC Class II, LCA and CD45R antigens were used for analysis of peripheral blood and lymph cells by flow cytometry. These MAbs were supplied lyophilized as ascitic fluid, and were reconstituted using 5 ml of sterile distilled water. To determine their optimal dilutions, MAbs were diluted two-fold from 1:100 to 1:800 and tested on sheep PBLs with a range of immunoconjugate dilutions. Most MAbs were found to be suitable for use at 1:200 dilution except anti-CD5 and anti-CD1 which worked optimally at 1:100 dilution. The optimal dilutions were chosen as the highest dilution which gave strong fluorescence with a clear distinction between positive and negative populations. All the MAbs were diluted to their optimum dilutions in IMFB, stored at 4°C and used within five days. The working dilution of GAM/Ig/FITC was determined by testing on sheep PBLs and optimal dilutions of several MAbs. A dilution of 1:80 was found to give strong fluorescence and little non-specific staining on control cells. DAS/IgG/FITC (SAPU) was used to identify SIg^+ cells by direct immunofluorescent staining. The optimal dilution was found to be 1:80 when tested on sheep PBLs. MAb SW73.2 (MHC Class II) was supplied as purified immunoglobulin at 1 mg/ml which had been conjugated with FITC for direct immunofluorescent staining of MHC Class II^+ cells. The working dilution of this conjugate was 1:100.

For two colour immunofluorescence, R-phycoerythrin conjugated rabbit anti-mouse IgG $F(ab')_2$ (RAM/IgG_1, $F(ab')_2$-RPE, Serotec, Oxford, England) was used. RPE stains positive cells red and like FITC, is excited by light at a wavelength of 488 nM. However, its emission spectrum can be distinguished from that of FITC. After titration of the immunoconjugate and testing on sheep PBLs and a range of MAbs, the optimum dilution of RAM-RPE was found to be 1:100.

3.8.2.2 Single colour immunofluorescence staining of cells for analysis by flow cytometry

The technique used for staining was similar to that employed for analysis by fluorescence microscopy. After staining and washing, cells were fixed in 1% paraformaldehyde and kept in the dark at 4°C until analyzed (Lanier and Warner, 1981).

3.8.2.3 Two-colour immunofluorescence staining of cells for analysis by flow cytometry

Expression of MHC Class II molecules on T lymphocyte subpopulations, $CD45R^+$ cells and $CD1^+$ cells was determined by two-colour immunofluorescence staining as described by Loken, Parks and Herzenberg (1977). Cells (10^6) were first incubated for 30 minutes at 4°C with 100 μl of optimal dilutions of MAbs specific for the respective subpopulations. After washing twice in IMFB, cells were incubated with 50 μl of 1:100 dilution of RAM-RPE for 30 minutes at 4°C. The labelled cells were again washed twice in IMFB before being incubated for 30 minutes at 4°C with SW73.2-FITC, an anti-MHC Class II MAb. After a final wash in IMFB to remove excess conjugate, cells were fixed in 1% paraformaldehyde and kept at 4°C in the dark until analyzed.

To identify SIg^+ lymphocytes expressing MHC Class II antigens, cells were incubated first with an anti-MHC II MAb (SBU-II), followed by RAM-RPE and then with DAS-FITC. The staining, washing and incubations of these cells was carried out as described above.

3.8.2.4 Flow cytometry

Cells were transferred from microtitre plates into small test tubes (RH-tubes, Sterilin), wrapped with aluminium foil and kept at 4°C in the dark until analyzed. Flow cytometry analysis of cells was performed using a fluorescence-activated cell analyzer (FACSCAN, Becton-Dickinson). The technique is similar to assessment of staining using fluorescence microscopy (Section 3.8.1) (Loken and Stall, 1982; Parks and Herzenberg, 1984). However, flow cytometry analysis allows a more accurate quantitation of fluorescent cell populations counting a total of 10,000 cells per sample. The FACSCAN also has an attached data storage and retrieval facility.

Cells in suspension pass from a reservoir into the centre of a nozzle sheath fluid which confines them to the centre of a liquid jet. As cells pass through a focused laser beam from an 488 nM argon ion laser they scatter laser light, the fluorescent molecules on the cells are excited and they fluoresce. The scattered light is collected by detector systems placed either in the direction of travel of the beam (forward-angle light scatter, or FSC) or at right angles to the direction of travel (90-degree or side light scatter or SSC) which is then converted into an electronic signal. Similarly, fluorescent emitted light which is shifted to a longer wavelength is then resolved from the original exciting wavelength of the laser (488 nM for FITC and RPE) by a combination of dichroic mirrors and long and short-pass filters which direct the light to a series of photomultiplier tube (PMT) detectors. The light scatter and fluorescence detector signals are then amplified and evaluated. The signal levels are digitised and stored in a computer for subsequent analysis.

3.8.2.5 Flow cytometry parameters

Data is usually derived by analyzing 10,000 cells (or events) per sample and FSC, SSC and fluorescent data are plotted as frequency histograms. The amount of light scatter in the forward angle is proportional to the volume of spherical cells (Mullaney, Van Dilla and Dean, 1969). Viable cells produce a larger degree of FSC than dead cells. This is useful in distinguishing live from dead cells (Loken and

Herzenberg, 1975; Traganos *et al.*, 1977); SSC is also proportional to cell volume but is affected by cell surface topography (for example cell villi), nucleus: cytoplasm ratio and heterogeneity of cell cytoplasm (size, number and optical properties of intracellular granules). Thus, neutrophils and lymphocytes with the same volume would give similar FSC signal but a different SSC. These differences in SSC and FSC are valuable in flow cytometry and are used to set electronic 'gates' for analysis of cells of certain size and complexity (Hudson and Hay, 1989).

3.8.2.6 Procedure for analysis of cells

The Consort 30 programme (Becton-Dickinson) was used in the flow cytometry analysis of cells. The cytometer was calibrated using control cells incubated with a 1:500 dilution of normal mouse serum and immunoconjugate only. It was found that the optimum settings for FSC and SSC were E01 and 326 respectively. Using the dot plot (Figure 3.4), gates were set to analyze the desired cell populations. The optimum sensitivities for FITC and RPE stained cells were obtained by observing and adjusting histogram profiles of gated cell populations. These were found to be 625 MV for FITC and 574 MV for RPE. For two-colour immunofluorescence analysis of cells, the cytometer was set at a compensation of nine to 30 MV to distinguish between the emission spectra of FITC and RPE.

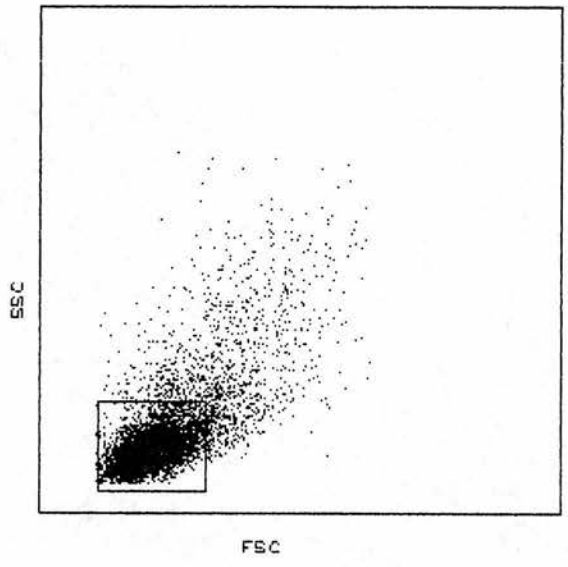
Single parameter data was presented as histograms (Figure 3.4). In these plots the degree of fluorescence or light scatter (channel number or fluorescence units) is presented on the x-axis and the frequency of cells on the y-axis. The scale on the x-axis is in logarithmic scale for fluorescence intensity and linear scale for light scatter. Figure 3.5 shows afferent cells stained for CD4 using specific MAb and fluorescein conjugated goat anti-mouse immunoglobulin. Results are expressed as percentage positive cells.

Contour plots were used to indicate the relationship of two cell qualities such as dual colour staining for two leucocyte antigens with FITC and RPE. These plots are similar to topographic maps, with increasing density of cells in a region

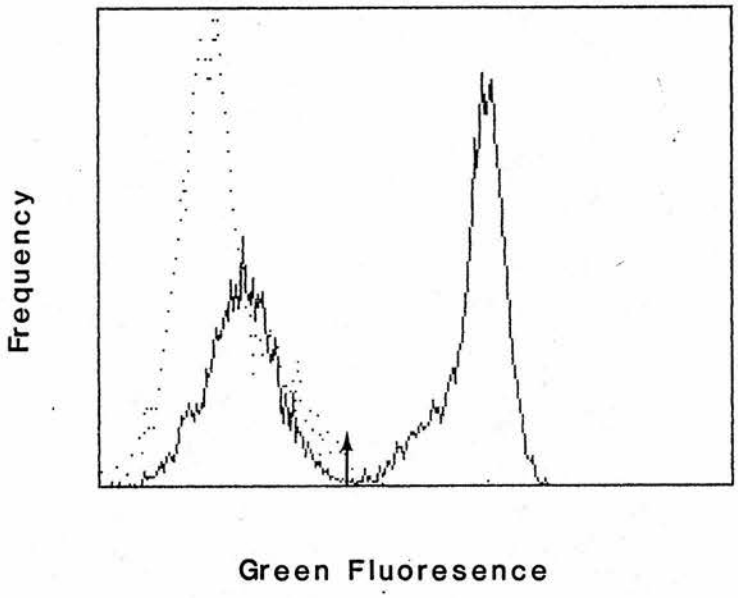
FIGURE 3.4 Dot plot; a two dimensional flow cytometry display of forward angle light scatter (FSC, x-axis) and side angle light scatter (SSC, y-axis) used to set 'gates' for analysis of sheep leucocyte populations. Light scatter parameters are on a linear scale.

FIGURE 3.5 Histogram showing a flow cytometry profile of relative fluorescence intensity of sheep efferent lymph cells stained for CD4 antigen. Cells were labelled with green fluorescence (x-axis) using anti-CD4 MAb and FITC conjugated goat anti-mouse Ig. The profile of $CD4^+$ stained cells (solid line) is bimodal indicating presence of two discrete populations. The arrow represents the marker between the positive and negative peaks. The dotted profile represents control cells incubated with normal mouse serum and GAM-FITC only. Fluorescence intensity is on a logarithmic scale.

3.4



3.5



represented by higher contour levels. The contour method chosen was the one which places contours so that a fixed fraction of cells is found in the area between adjacent contours. For analysis, the contour maps were divided into quadrants by setting markers. The negative control cell population is present in the lower left quadrant while the double stained cell population is in the upper right quadrant. Percentage positive cells were obtained from each quadrant as indicated in Figure 3.6.

3.9 Immunohistochemical Staining of Frozen Tissue Sections

3.9.1 Monoclonal antibodies and immunoperoxidase conjugates

MAbs identifying CD5, CD4, CD8, SBU-T19, MHC Class II, CD45R, CD1, VPM32 and VPM33 were used to localize respective cellular phenotypes on frozen tissues. Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (GAM/IgG (H+L)/HRPO, Nordic Immunological Laboratories, Tilburg, Netherlands), was supplied as a lyophilized product. It was reconstituted with 1 ml of sterile distilled water, centrifuged to remove insoluble particles, divided into small aliquots of 100 μl each and stored at or below -25°C wrapped in aluminium foil. Before use, an aliquot was slowly thawed in the dark at ambient temperature, centrifuged again and then used to prepare working dilutions by addition of the required amount of PBS, pH 7.2.The optimal working dilutions of both MAbs and immunoconjugate were determined by titration on cryostat sections of skin and lymph nodes from healthy, normal sheep (Tables 3.2 and 3.3). Doubling dilutions from 1:40 to 1:320 in immunoperoxidase buffer (IMPB) containing 2% normal sheep serum, 0.1% BSA and 0.08% Tween 80 in PBS pH 7.2 (Appendix II) were prepared. MAbs were used as undiluted tissue culture supernatants and at 1:2, 1:4 and 1:8 dilutions. Dilutions of MAbs and immunoconjugates were chosen which gave high specific staining.

3.9.2 Immunohistochemical staining procedure

Frozen tissues kept at -70°C were mounted on cold specimen blocks using OCT embedding compound (Miles Labs. Ltd., Slough, England) and 5 μm thick sections cut using a cryostat (Bright Inst. Co. Ltd., Huntingdon, England) at -39°C.

Results

Table 3.2 **Titration of MAbs for immunohistochemical staining. Results indicate the intensity of staining (and background non-specific staining). A dilution of 1:160 of immunoconjugate was used**

MAb	Dilutions of MAbs			
	Undiluted	1:2	1:4	1:8
	a b			
SBU-T1	$2^+(2^+)$	$2^+(1^+)^*$	$1^+(1^+)$	$1^+(1^+)$
SBU-T4	$4^+(2^+)$	$2^+(1^+)$	$2^+(1^+)^*$	$1^+(1^+)$
SBU-T8	$4^+(3^+)$	$3^+(2^+)$	$2^+(1^+)^*$	$1^+(2^+)$
SBU-T19	$3^+(2^+)$	3 (1^+)	$3^+(1^+)^*$	$1^+(1^+)$
SBU-T6	$3^+(3^+)$	$2^+(2^+)$	$2^+(1^+)^*$	$1^+(1^+)$
SBU-LCAp220	$4^+(2^+)$	$3^+(1^+)$	$3^+(1^+)^*$	$2^+(1^+)$
SBU-II	$4^+(3^+)$	$3^+(2^+)$	$2^+(1^+)^*$	$1^+(1^+)$
VPM32	$3^+(3^+)$	$2^+(2^+)^*$	$1^+(2^+)$	$1^+(2^+)$
VPM33	$3^+(2^+)$	$2^+(2^+)$	$2^+(1^+)^*$	$1^+(1^+)$

a - Intensity of specific staining (1^+ - low intensity, 4^+ - high intensity staining)
b - Background staining (1^+ - low background, 3^+ - high background)
* The highest dilution of MAb with a staining score of 2^+ or more and highest staining/background ratio was chosen

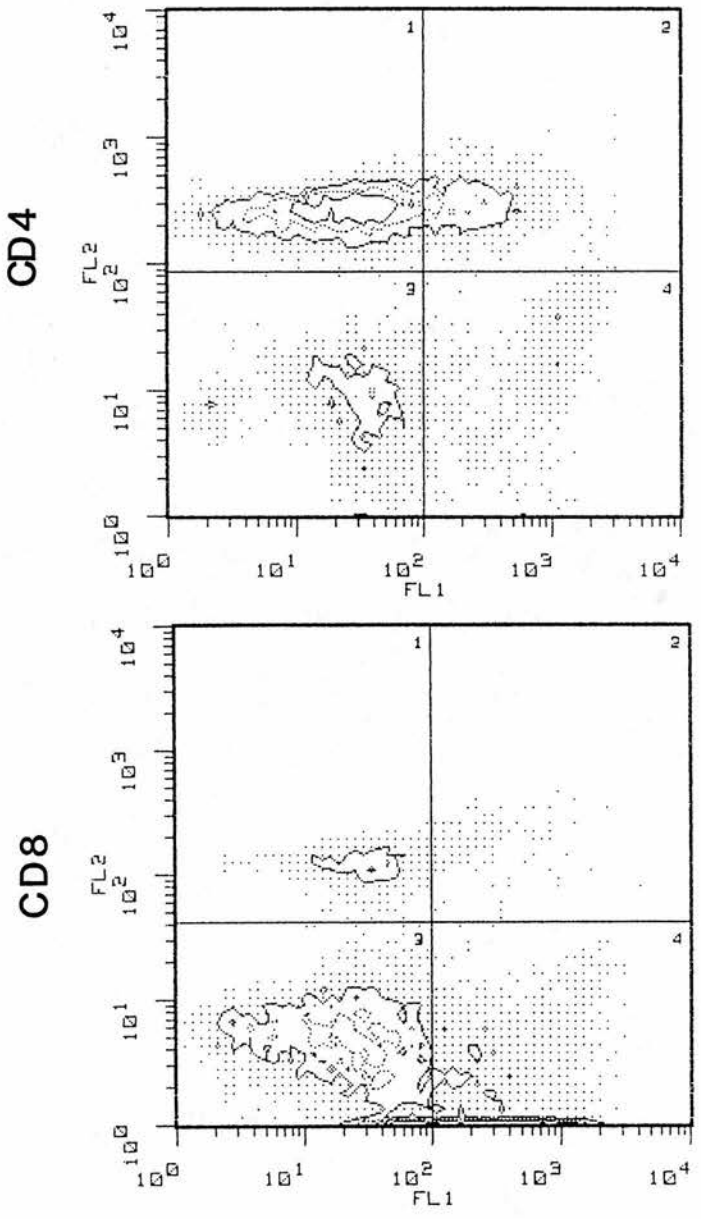
TABLE 3.3 Titration of HRPO conjugates using MAbs diluted at optimal concentrations

Immunoconjugate dilution	MAbs SBU-T1	SBU-T4	SBU-LCAp220	SBU-II
	a b			
1:40	$3^+(2^+)$	$4^+(2^+)$	$4^+(2^+)$	$4^+(3^+)$
1:80	$3^+(1^+)^*$	$4^+(2^+)^*$	$3^+(1^+)$	$3\ (2^+)^*$
1:160	$2^+(1^+)$	$3^+(2^+)$	$2^+(1^+)^*$	$2^+(2^+)$
1:320	$1^+(1^+)$	$2^+(1^+)$	$2^+(1^+)$	$1^+(1^+)$

a - Intensity of specific staining (1^+ - low intensity staining, 4^+ high specific staining)
b - Background staining (1^+ - low background staining, 3^+ high background staining)
* Three of the four MAbs stained better and clearer at conjugate dilution of 1:80 which was subsequently used in immunohistochemical staining

FIGURE 3.6 Two colour immunofluorescence analysis of sheep efferent $CD4^+$ and $CD8^+$ lymphocytes for MHC Class II antigen expression. $CD4^+$ and $CD8^+$ cells were labelled with specific MAbs and RPE conjugated rabbit anti-mouse Ig, giving red fluorescence. MHC Class II^+ cells were labelled with FITC conjugated anti-MHC Class II MAb (Sw73.2) giving green fluorescence. The results are represented as contour plots with fluorescence intensity on a logarithmic scale. Contour plots are divided into four quadrants. The upper right quadrants (2) represents cells which express both CD4 or CD8 and MHC Class II antigens.

3.6



Sections were placed on multispot slides (C.A. Hendley (Essex) Ltd., Essex, England), air dried for one hour and then fixed in cold acetone for five minutes. Endogenous peroxidase was blocked by washing the slides twice for 15 minutes each in 0.3% hydrogen peroxide (BDH, Poole, England) in PBS pH 7.2. Sections were again washed for 10 minutes in two changes of PBS for 10 minutes and once for 15 minutes in IMPB. Fifty microlitres of MAb diluted 1:4 in IMPB was applied onto the tissue sections which were then incubated at room temperature overnight in moist chambers. As controls, tissue sections were incubated with normal mouse serum diluted 1:500. After the sections were washed once each in PBS and IMPB for 10 minutes they were incubated with GAM/IgG/HRPO conjugate diluted 1:80 for one hour at room temperature in moist chambers. Sections were then washed in PBS for 10 minutes, and developed in diaminobenzidine tetrahydrochloride (DAB, BDH, Appendix II) containing 0.01% H_2O_2 for 10 minutes. The developed sections were washed twice in distilled water for 10 minutes and the staining reaction enhanced by placing the slides in a solution of 0.01% osmium tetroxide in PBS (TAAB Labs, Reading, England) for one minute. After a further rinse in distilled water, sections were lightly counterstained for 30 seconds in Mayer's haematoxylin and then washed in running tap water for five minutes. Sections were dehydrated in graded alcohols (70%, 90%, Absolute, Absolute), cleared in xylene and then mounted using Depex mounting medium (BDH). Slides were examined by light microscopy using a Leitz Laborlux at x10 and x40 eyepiece. Photographs were taken on ILFORD Pan-F 35 mm film or AGFA 50 RS colour (35 mm).

CHAPTER FOUR
CELLULAR PHENOTYPE DYNAMICS IN LOCAL SKIN REACTIONS FROM SHEEP INFECTED WITH METACYCLIC FORMS OF *T. CONGOLENSE*

4.1 Introduction

The sequential histopathology of local skin reactions elicited in mammalian hosts by various trypanosome species has been described by a number of authors (Emery and Moloo, 1980, 1981; Gray and Luckins, 1980; Akol and Murray, 1982; Dwinger *et al.*, 1987a). In hosts infected with *T. congolense*, little or no histological changes occur in the skin within the first five days after infective tsetse fly bite. However, within seven days, the development of local skin reactions is characterized by an intense inflammatory response and invasion of the dermal collagen by trypanosomes. The skin reaction is manifested by an increase in total cellularity, congestion, tissue oedema and necrotic foci at all levels of the skin. Trypanosomes are present in the skin five days after infection as demonstrated by subinoculation into mice with fluid expressed from the lesion (Akol and Murray, 1982; Dwinger *et al.*, 1987). By seven days, trypanosomes are evident by microscopical examination of expressed fluid or histological sections. Many trypanosomes are present in the skin, especially in dilated lymphatics of papillary dermis and hypodermis during the peak of reactions. The numbers of trypanosomes then decline to undetectable levels 50 days after infection (Gray and Luckins, 1980; Akol and Murray, 1982). The intensity, distribution, and type of cellular infiltrate varies during the course of development and regression of local skin reactions caused by infection with *T. congolense* (Emery and Moloo, 1980; Gray and Luckins, 1980; Akol and Murray, 1982). During the latent phase (up to five days), the infected tsetse fly bite site contains no detectable cellular infiltrate. The acute inflammatory phase (six to nine days), is characterized by a diffuse infiltrate of polymorphonuclear cells (PMNs) and lymphocytes in the dermal papillae, hypodermis, around blood vessels, sweat glands and hair follicles. The cellular infiltrate at the peak of the skin reaction (10 to 14 days), consists mainly of mononuclear cells. The declining phase (from 14 days) although characterized by a decrease in all cell types shows a marked blastogenic response of lymphoid cell populations, with large lymphocytes and mitotic figures. The peak of this blastogenic response occurs 18 days after infection. Plasma cells and few macrophages are

present during this period. Thereafter, the intensity of cellular infiltrate declines leaving only foci of small and medium lymphocytes in perivascular areas (Akol and Murray, 1982).

The development of trypanosome induced local skin reactions is important in both the pathogenesis of the disease and the induction of protective immune responses to cyclically transmitted trypanosomes (Taiwo *et al.*, 1990). However, the course of development is influenced by other factors, including the immune status of the host, concurrent trypanosome infections and trypanocidal therapy (Akol and Murray, 1985; Dwinger *et al.*, 1987b). Cattle infected with *T. congolense* and treated 10 days later do not develop protective immunity. Concurrent trypanosome infections interfere with development of local skin reactions and establishment of secondary infections (Luckins and Gray, 1983; Dwinger *et al.*, 1989). In order to understand the mechanisms involved in these processes, it is necessary to obtain a more detailed knowledge of the cellular responses which occur in the local skin reaction during the early stages of infection.

The purposes of the experiments described in this chapter were three-fold; firstly, to determine the cellular changes occurring in the skin before clinical development of the local skin reactions using light and electron microscopy. Secondly, to examine the sequential histological and immunocytochemical changes, especially the dynamics of cellular phenotypes infiltrating local skin reactions and, finally, to examine the effects of trypanocidal therapy and concurrent trypanosome infections in sheep on the cellular phenotype dynamics at sites inoculated with homologous or heterologous *T. congolense* serodemes.

4.2 Materials and Methods

4.2.1 Trypanosomes and establishment of infection

Sheep were infected by intradermal inoculation on their shaven flanks of 0.2 ml PSG containing 2 x 10^5 culture-derived metacyclic forms of *T. congolense*

TREU 1457, 1881 or 1885. The inoculation sites were then clearly marked with a black felt-tipped pen.

4.2.2 Morphometric analysis of lymphocyte subsets

In sections stained using specific MAbs and immunoperoxidase technique, (Section 3.9), numbers of $CD5^+$, $CD4^+$. $CD8^+$. SBU-$T19^+$ and $CD45R^+$ cells per unit area were determined using a modification of the method used by Armstrong *et al.* (1987) and Gorrell, Miller and Brandon (1988a). For each phenotype, cells in the diffuse infiltrate were counted at x400 magnification in at least five similar fields (each 0.08 mm^2). At least four sections of each skin sample were counted using a graticuled eyepiece with 121 intersections (Periplan GF, Leitz Wetzlar, Germany). Cells in very dense clusters were not counted. Cells expressing MHC Class II^+ and FCR^+ were difficult to visualize distinctly in the skin and were thus not counted. The median values and ranges of the cell counts in the chancre and the ratios of numbers of $CD4^+$ and $CD8^+$ cells were determined.

4.2.3 Transmission electron microscopy

Skin samples for transmission electron microscopy (TEM) were cut into one millimeter cube (1 mm^3) pieces and were immediately fixed in 3% glutaraldehyde solution in 0.1M sodium cacodylate buffer (pH 7.3) for two to three hours (Sabatini *et al.*, 1963). Fixed tissues were washed three times in 0.1 M cacodylate buffer for 20 minutes each. The specimens were post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 45 minutes (Sabatini *et al.*, 1963), washed twice in cacodylate buffer for 15 minutes each and then stored at 4°C overnight. After washing three times in distilled water for 20 minutes, the specimens were dehydrated by placing in 50%, 70% and 90% acetone for 10 minutes each and then three times for 10 minutes each in 100% acetone (Hayat, 1970). Tissues were infiltrated using two changes of 50:50 araldite:analar acetone for 30 minutes and overnight at 60°C, and then in araldite mix three times for one hour each. A final infiltration step was carried out in 19:1 araldite: accelerator in two changes for one hour each before

embedding in araldite mix/accelerator (19:1) for 48 hours at 60°C (Glauert and Glauert, 1958). Ultrathin sections (60 μ) were cut on a diamond knife with a Reichert OMU4 ultramicrotome and mounted on 200 mesh copper grids. Sections were stained in uranyl acetate in 50% ethanol for 30 minutes and in lead citrate (Reynolds, 1963) for five minutes. Stained sections were viewed and photographed using a Philips 400 Transmission Electron Microscope.

4.2.4 Experimental design

Experiment 1. **Light and electron microscopical examination of the inoculation site prior to the clinical development of the local skin reaction**

In order to determine the cellular changes occurring at the inoculation site prior to clinical development of the local skin reaction, four Suffolk sheep were each infected at eight sites (four on each flank) with *T. congolense* TREU 1457. Skin samples were obtained on days three and five after infection for light and transmission electron microscopical examination.

Experiment 2. **Determination of cellular phenotype dynamics in local skin reaction in sheep during primary *T. congolense* infection**

Four sheep were inoculated sequentially with TREU 1885 at six sites, on their flanks, on days 0, 1, 3, 6, 7 and 8. Five days after the last injection, the sheep were killed to obtain local skin reactions at various stages of development between five and 13 days post-infection. Four other sheep were inoculated intradermally at six sites and skin biopsies from skin reactions which developed were removed under local anaesthesia on days 12, 13, 15, 21 and 30 days post-infection.

Experiment 3. **Effect of trypanocidal drug treatment on the development of local skin reactions**

Two sheep were inoculated at eight sites, four on each flank with TREU 1885. The sheep were treated with a single dose of 7 mg/kg diminazene aceturate (Berenil, Hoechst, FRG) by intramuscular injection five days after infection, just before the time that local skin reactions would normally be detectable by inspection and palpation. Two skin biopsies were taken from each of the sheep 12, 17, 26 and 30

days after infection. In order to examine the effects of challenge infection on cellular dynamics in recovered animals the sheep were then challenged two weeks after treatment with the homologous (TREU 1885) and two heterologous serodemes (TREU 1457 and 1881) at two different sites each. Skin biopsies were removed from inoculation sites on day seven after challenge.

Experiment 4. **Effects of concurrent trypanosome infection on cellular phenotype dynamics**

Two sheep were infected with TREU 1885 and 30 days later, superinfected with TREU 1885, TREU 1457 and TREU 1881 on two different sites each. Skin samples were taken from each of the sites of secondary challenge on day seven post-infection.

4.3 Results

4.3.1 Light and electron microscopical observations prior to clinical development of local skin reaction

By three days after infection, trypanosomes could be detected in the dermal collagen and in the interstitial spaces but there was little evidence of any changes in the collagen. On day five, trypanosomes were present in the dermal lymphatics (Figure 4.1) and although some degenerating parasites could be observed in the interstitial spaces trypanosomes were observed in close proximity to macrophages and there was no evidence of phagocytosis. A few inflammatory cells were seen in the collagen bundles near the trypanosomes and disintegration of collagen and oedema was evident. Mast cells from the skin of sheep five days after infection with *T. congolense* showed marked changes compared to uninfected skin. These mast cells were smaller, had numerous cytoplasmic processes and the cytoplasm contained fewer electron dense granules or vacuoles with less dense granule matrix (Figure 4.2).

4.3.2 Local skin reactions in sheep during primary infection with *T. congolense*

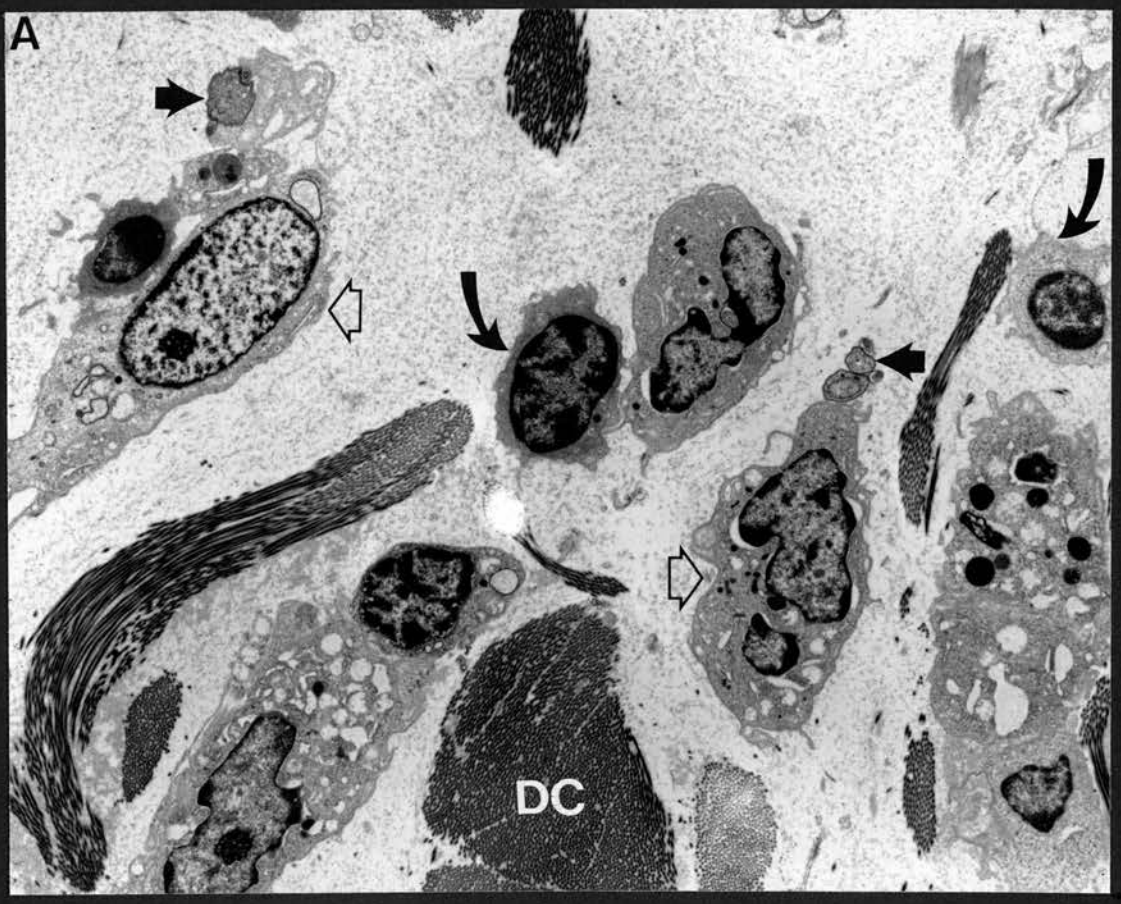
4.3.2.1 Clinical development

Intradermal inoculation of metacyclic forms of *T. congolense* TREU 1885, induced development of local skin reactions at all inoculation sites in normal sheep.

FIGURE 4.1 Transmission electron micrographs of the skin of sheep, five days after intradermal inoculation of culture derived metacyclic forms of *Trypanosoma congolense*.

(A) Trypanosomes (solid arrows) in close proximity to macrophages (open arrows) in the dermis. Lymphocytes (curved arrow) are also present. DC, dermal collagen (X2150).

(B) A dermal lymphatic containing several trypanosomes (solid arrows), lymphocyte (open arrow) and degenerating host leucocyte (star). EC, endothelial cell lining the dermal lymphatic (X2150).


A
DC

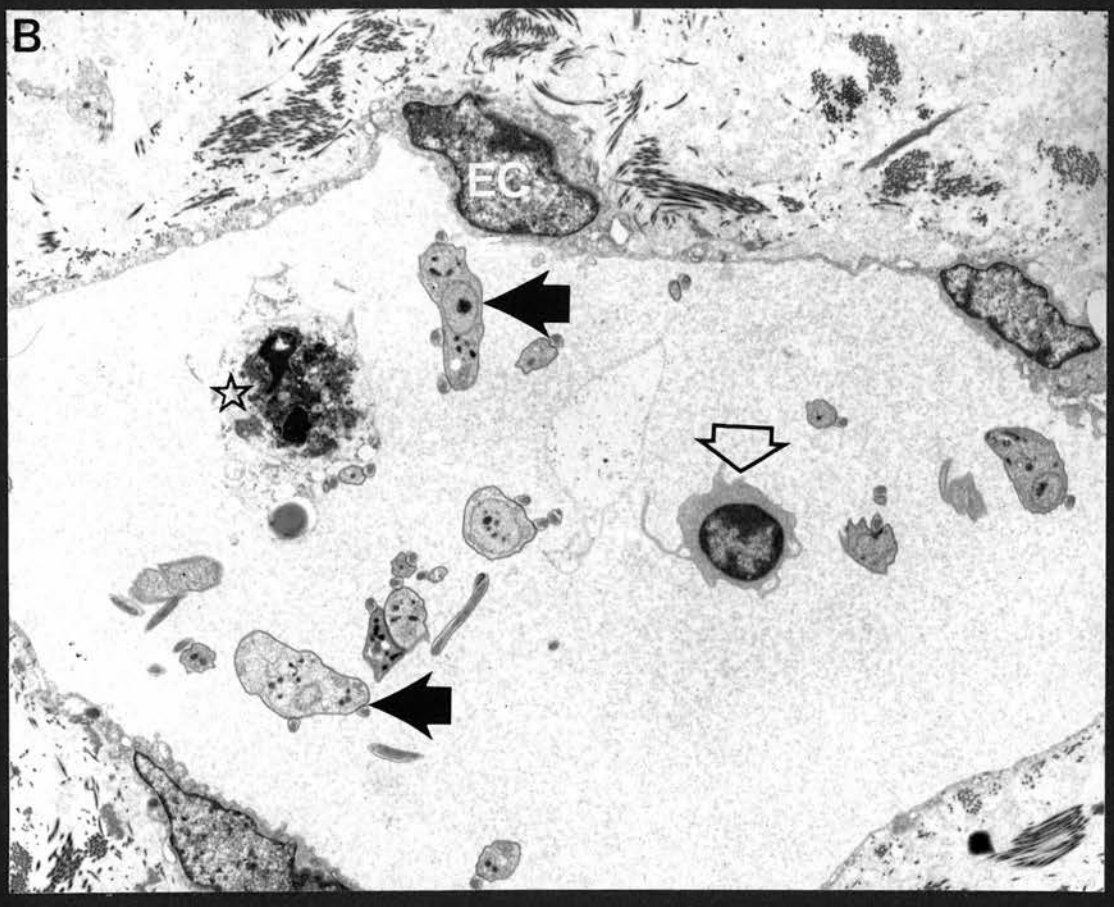
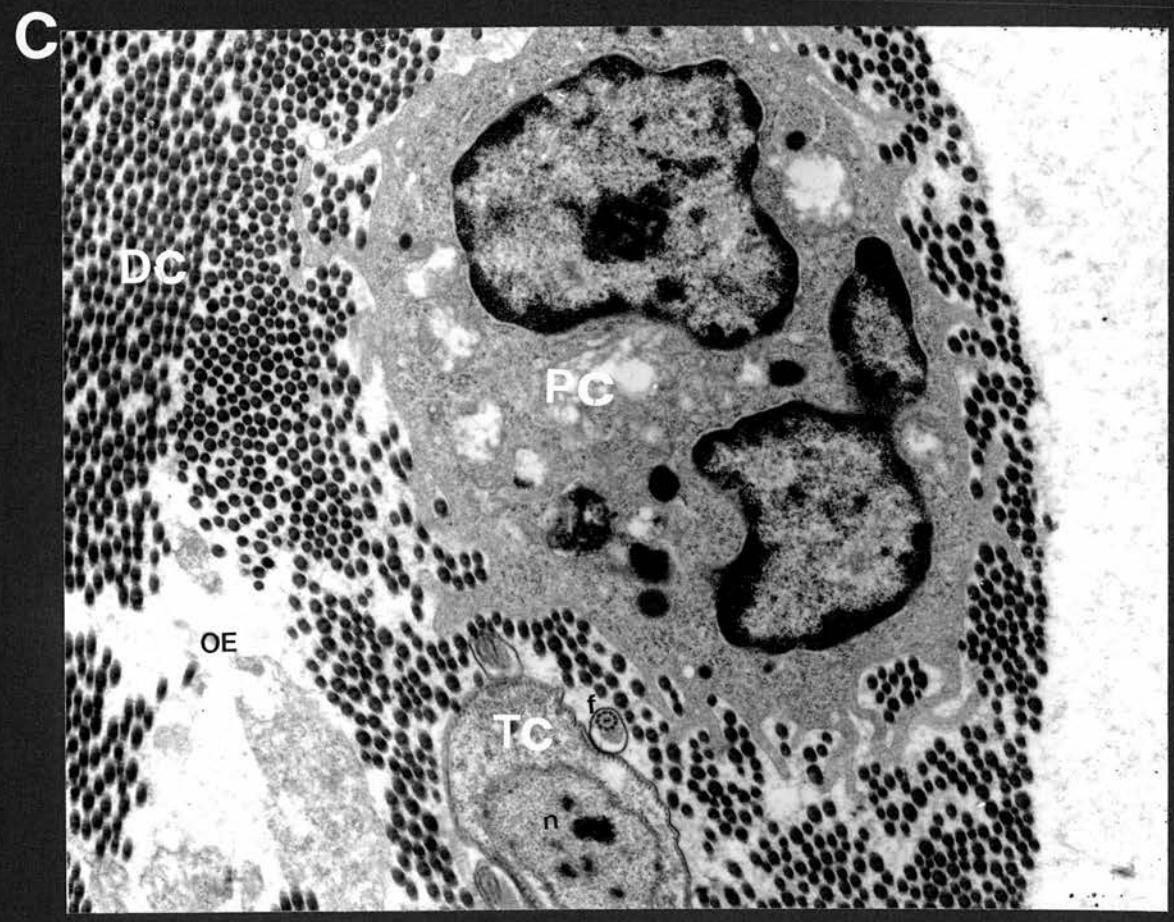

B
EC

FIGURE 4.1 Transmission electron micrographs of the skin of sheep, five days after intradermal inoculation of culture-derived metacyclic forms of *T. congolense*.

(C) *Trypanosoma congolense* (TC) and phagocytic cell (PC) in dermal collagen. DC, dermal collagen; f, flagella; OE, oedema; n, nucleus (X7700).

(D) Trypanosomes (arrows) in the dermal collagen surrounded by a clear area. A host macrophage (arrowed) is present between the collagen bundles, and the dermis contains extensive oedema (OE) (x3550)


C
DC
PC
OE
TC
f
n

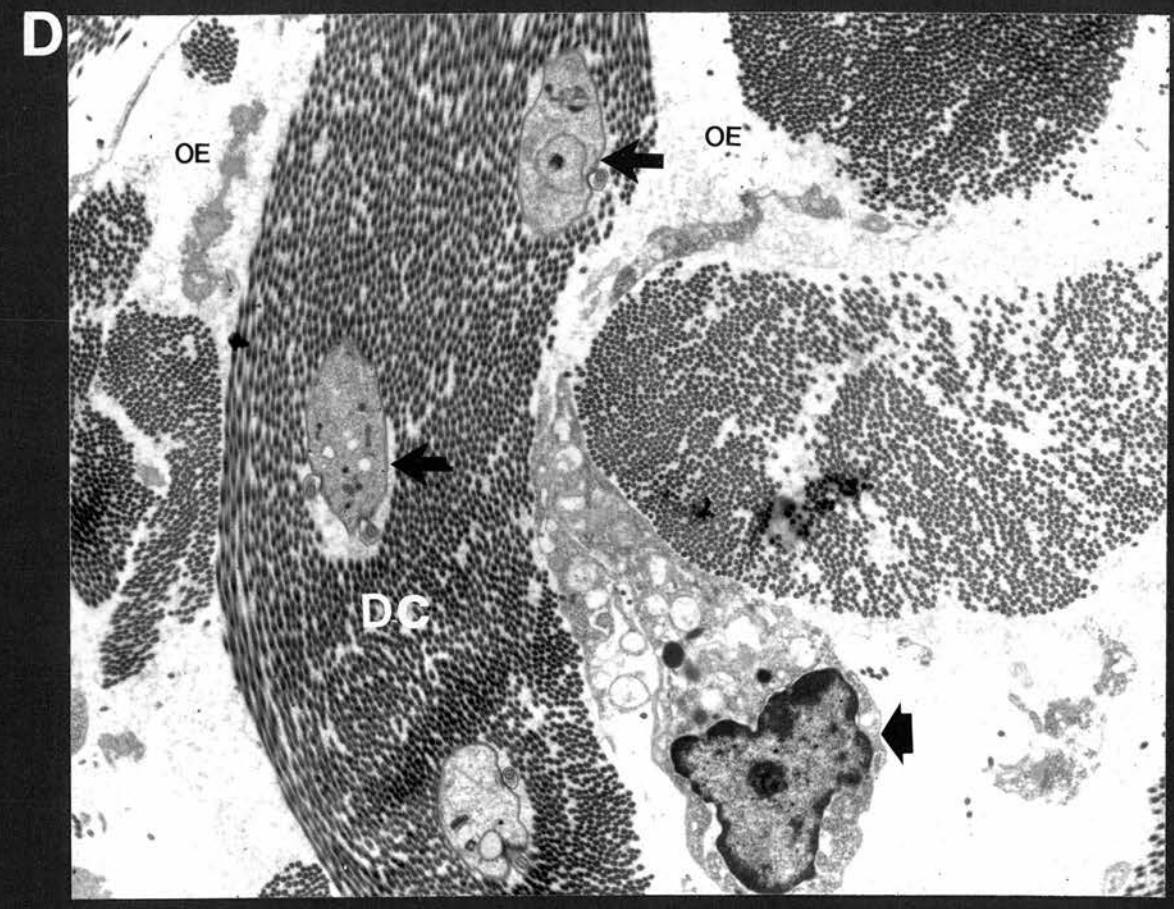

D
OE
OE
DC

FIGURE 4.2 Transmission electron micrographs of mast cells in the skin of sheep following inoculation of culture-derived metacyclic forms of *T. congolense*.

(A) Mast cells (solid arrows) in the skin of uninfected sheep. Numerous electron dense granules are present in the cytoplasm. The skin contains a few neutrophils (open arrow), mononuclear cells (MC) and fibroblasts (f) (X2150).

(B) Mast cell (solid arrow) from the skin of sheep 5 days after infection. The mast cell contains fewer electron dense granules, empty channels and numerous extended processes. L, lymphocytes, neutrophils (open arrows), f, fibroblasts (X2750).

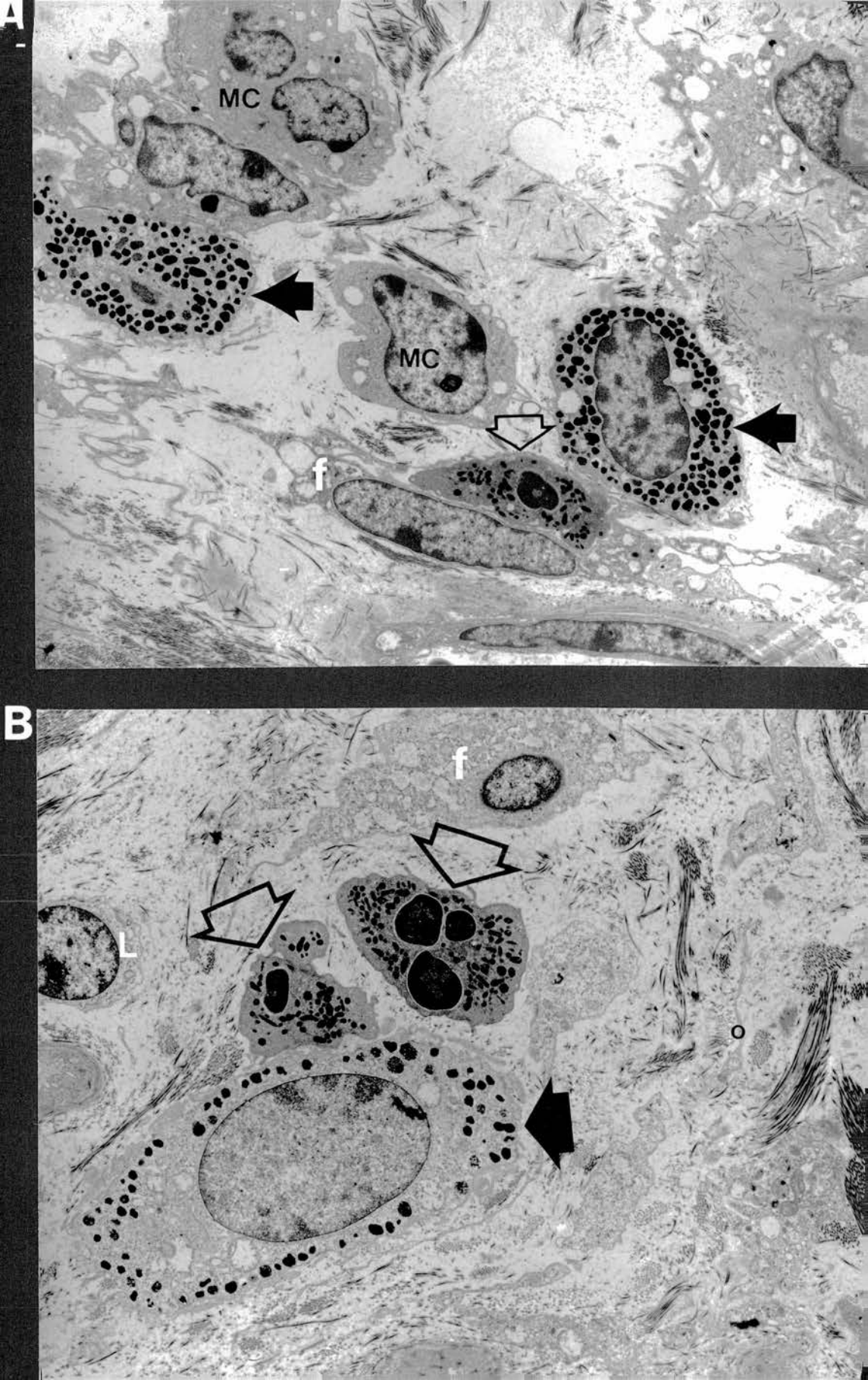
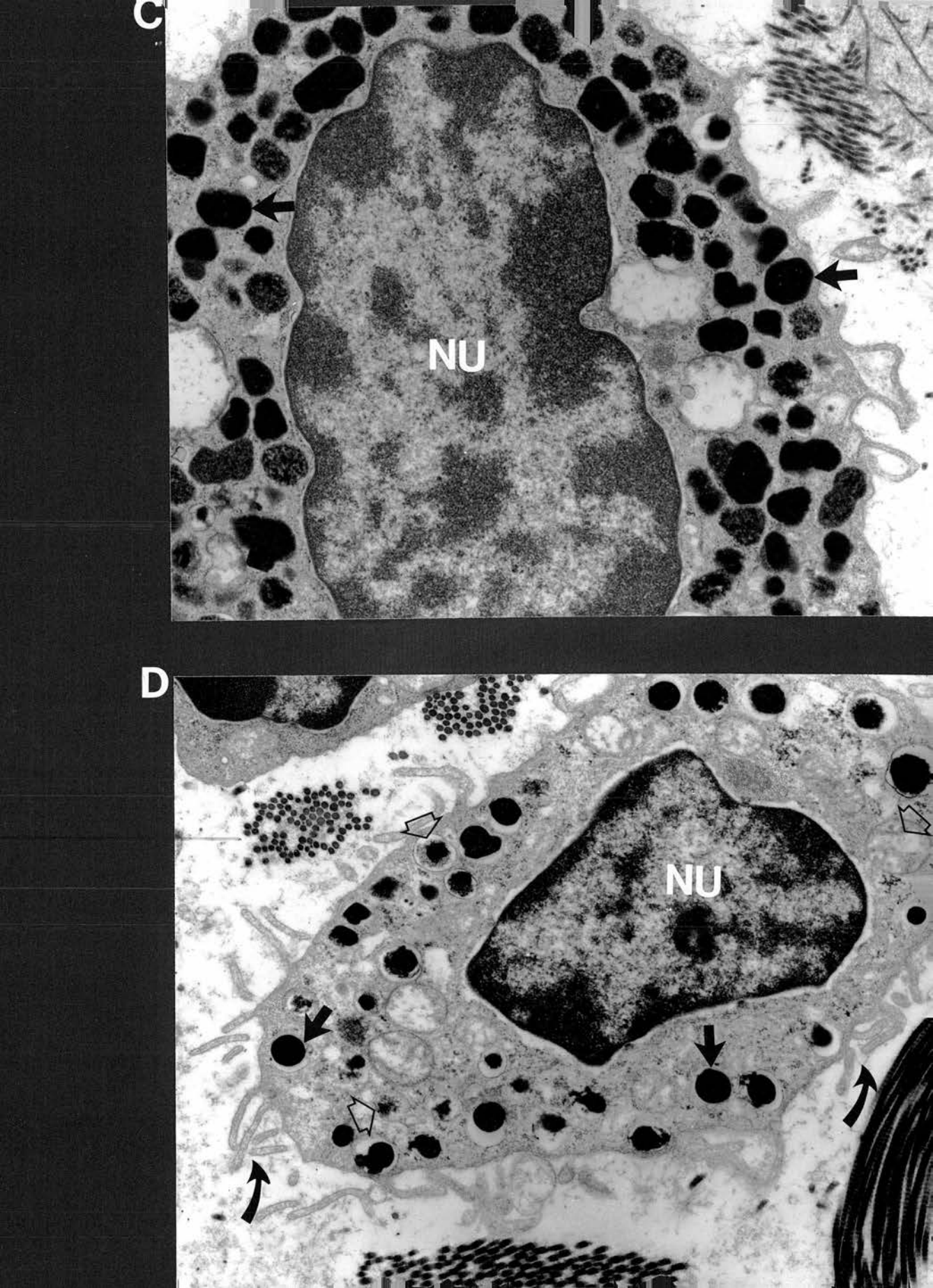

A
MC
MC
f
B
f
L
O

FIGURE 4.2

(C) Mast cells in the skin of uninfected sheep showing numerous electron dense granules in the cytoplasm (arrows). NU, nucleus (x10000).

(D) Mast cell in the skin of sheep five days after infection. Few electron dense granules are present in the cytoplasm. Some cytoplasmic vacuoles contain less dense granule matrix (open arrows). The mast cell also shows numerous cytoplasmic processes (curved arrows). NU, nucleus (x10000).


C
NU
D
NU

The appearance of these local skin reactions was similar to that reported by other workers (Gray and Luckins, 1980; Akol and Murray, 1982; Dwinger *et al.*, 1987a). Skin thickness increased from a pre-infection mean value of 3 mm to 6.3 mm by six days after infection (Figure 4.3) and then regressed gradually to below 4 mm 17 to 30 days after infection when skin reactions were no longer detectable macroscopically.

4.3.2.2 Histopathology

Sections from normal skin contained very few leucocytes scattered in the dermis and hypodermis. Between five to seven days after infection local skin reactions were characterized by infiltration of numerous polymorphonuclear leucocytes (PMNS) and mononuclear cells in the papillary dermis, around the vascular trunks, reticular dermis and hypodermis (Figure 4.4 and Figure 4.5). At the peak of the cellular reaction (10 to 15 days after infection), the cellular infiltrate comprised mainly of lymphocytes, lymphoblasts, macrophages, plasma cells and very few neutrophils (Figure 4.5). From 17 to 30 days after infection the cellular infiltrate consisted of lymphoblasts, plasma cells, macrophages and fibroblast-like cells which decreased progressively in density.

4.3.2.3 Cellular phenotypes

Representative illustrations of the changes in cellular phenotypes in local skin reactions are shown in Figure 4.6 and quantitative morphometric analyses in Table 4.1. In order to simplify the analysis and presentation of results, the observations on individual reactions were pooled into five separate time periods depending on the time after infection, skin thickness and histological appearance of the skin reaction. These periods were designated as one (day 0, normal skin), two (day five to seven, peak skin thickness with dense infiltrate of mononuclear cells and PMNS), three (day 10 to 15, regressing skin thickness but dense mononuclear cell infiltrate), four (day 17 to 23 regressed chancres, with moderate cellular infiltrate) and, five (day 26 to 30 regressed chancre with low cellular infiltrate).

TABLE 4.1 Median values (and ranges) of lymphocyte phenotypes in the skin reaction of sheep infected with *T. congolense*.

Lymphocyte phenotype	Days after infection (periods) 0 (1)	5-7 (2)	10-15 (3)	17-23 (4)	26-30 (5)
CD5	5*(1-28)	103.5(43-239)	110.5(41-361)	70.0(44-110)	32.0(5-119)
CD4	4(1-15)	83.0(20-188)	86.0(20-365)	41.5(13-89)	30.5(4-74)
CD8	6(3-16)	45.0(9-129)	60.5(8-114)	59.0(10-135)	42.0(8-146)
SBU-T19	2(0-9)	6.0(2-89)	6.0(0-13)	4.0(0-19)	5.0(1-21)
CD45R	0(0-6)	108.0(32-177)	33.5(0-155)	53.0(34-76)	10.0(0-69)
CD4/CD8	1.13(0.5-1.29)	2.07(1.01-4.77)	1.44(0.39-3.5)	0.74(0.5-1.19)	0.78(0.56-0.9)

*Number of cells per 0.08 mm² field (x40 objective, x10 eyepiece)

4.3

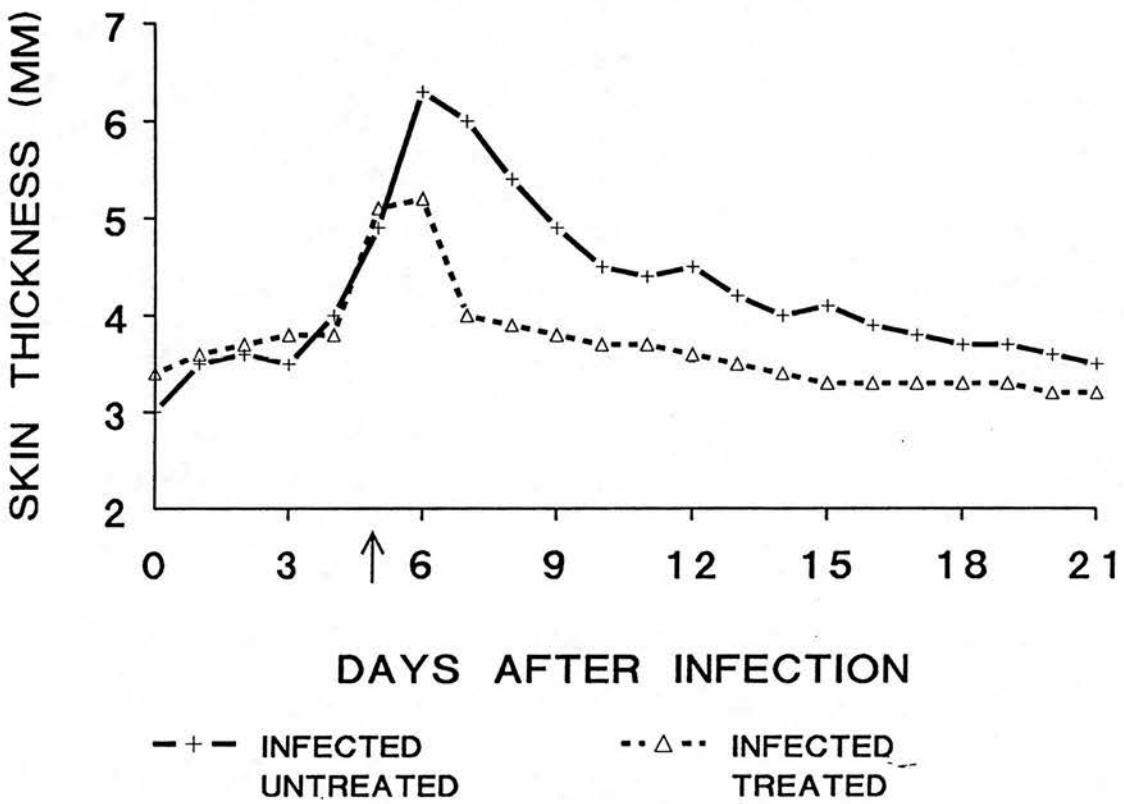

SKIN THICKNESS (MM)
7
6
5
4
3
2
0
3
6
9
12
15
18
21
DAYS AFTER INFECTION
INFECTED
UNTREATED
INFECTED
TREATED

FIGURE 4.3 Kinetics of development of local skin reactions as measured by mean thickness (mm) of the skin at sites of inoculation with metacyclic forms of *T. congolense* TREU 1457. The graph also indicates the effect of trypanocidal therapy at day five (arrow) on the development of the local skin reactions.

4.3

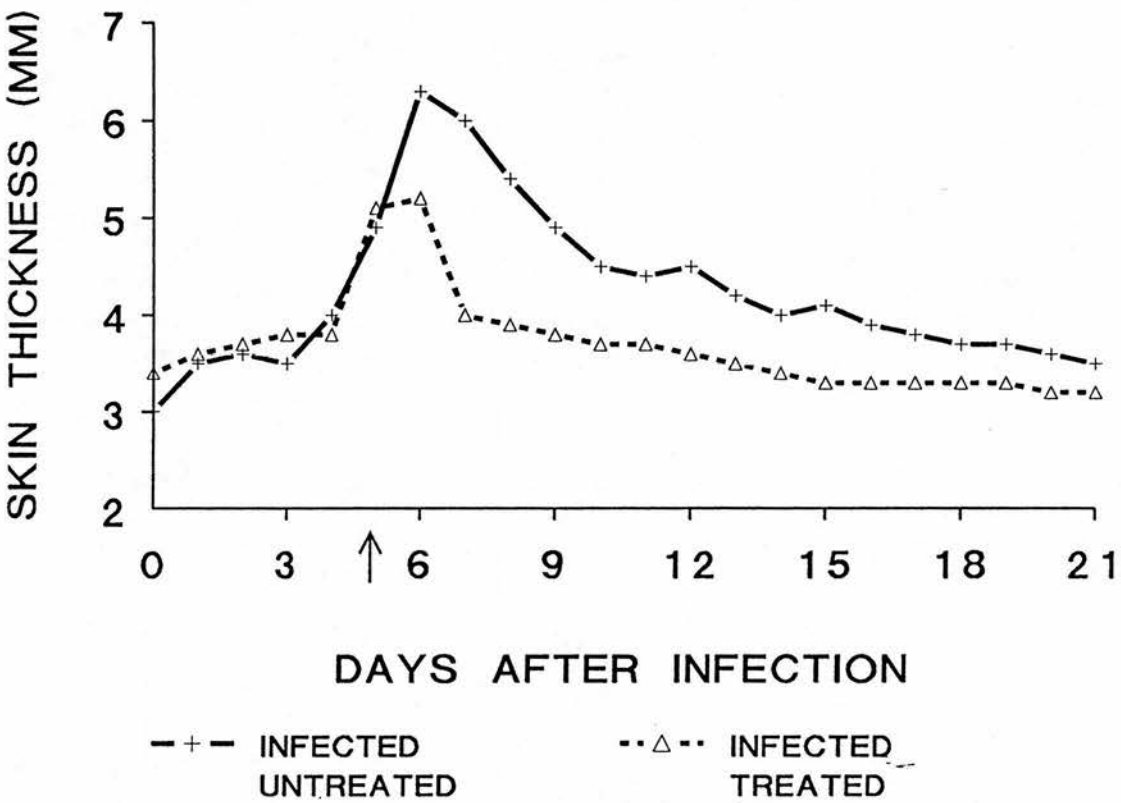
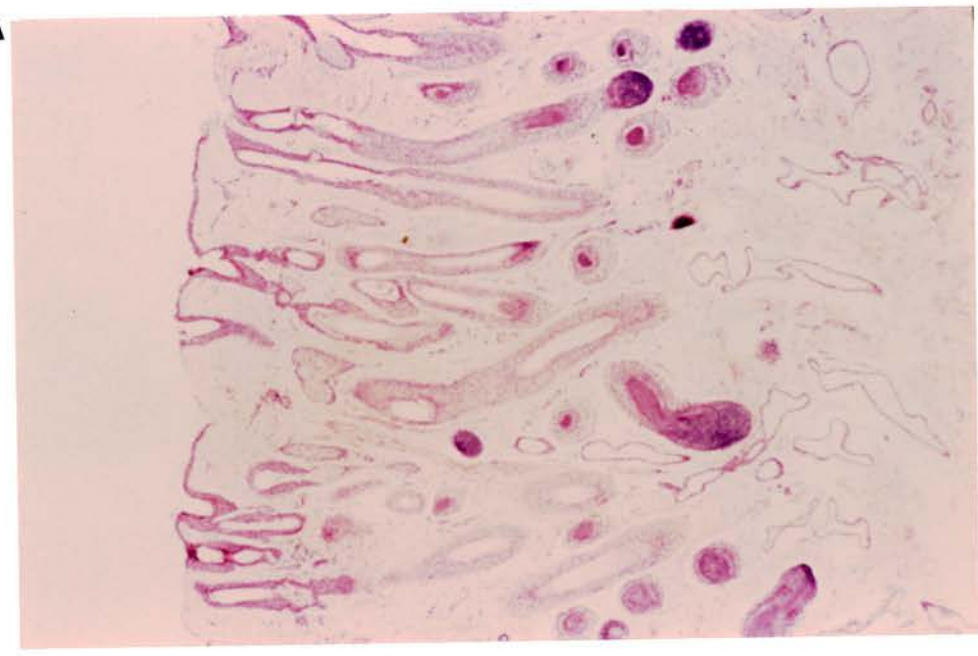

SKIN THICKNESS (MM)
7
6
5
4
3
2
0
3
6
9
12
15
18
21
DAYS AFTER INFECTION
INFECTED UNTREATED
INFECTED TREATED

FIGURE 4.4

(A) Histological section through uninfected ovine skin. H + E x50.

(B) Histological section of ovine skin seven days after intradermal inoculation of culture-derived metacyclic forms of *T. congolense* TREU 1457 showing intense cellular infiltration in the dermis H + E x50.

A



B

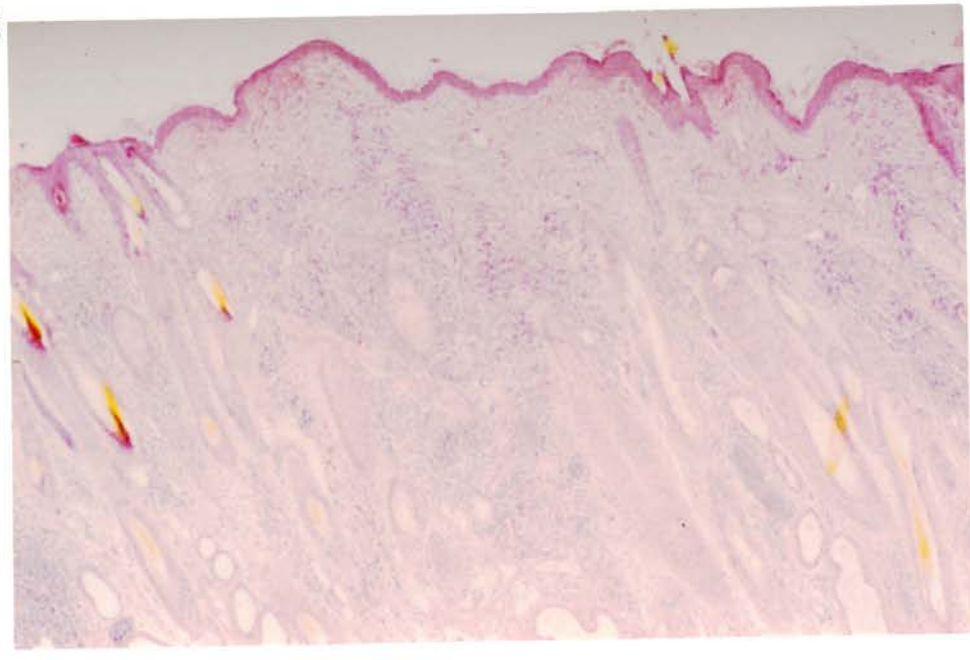
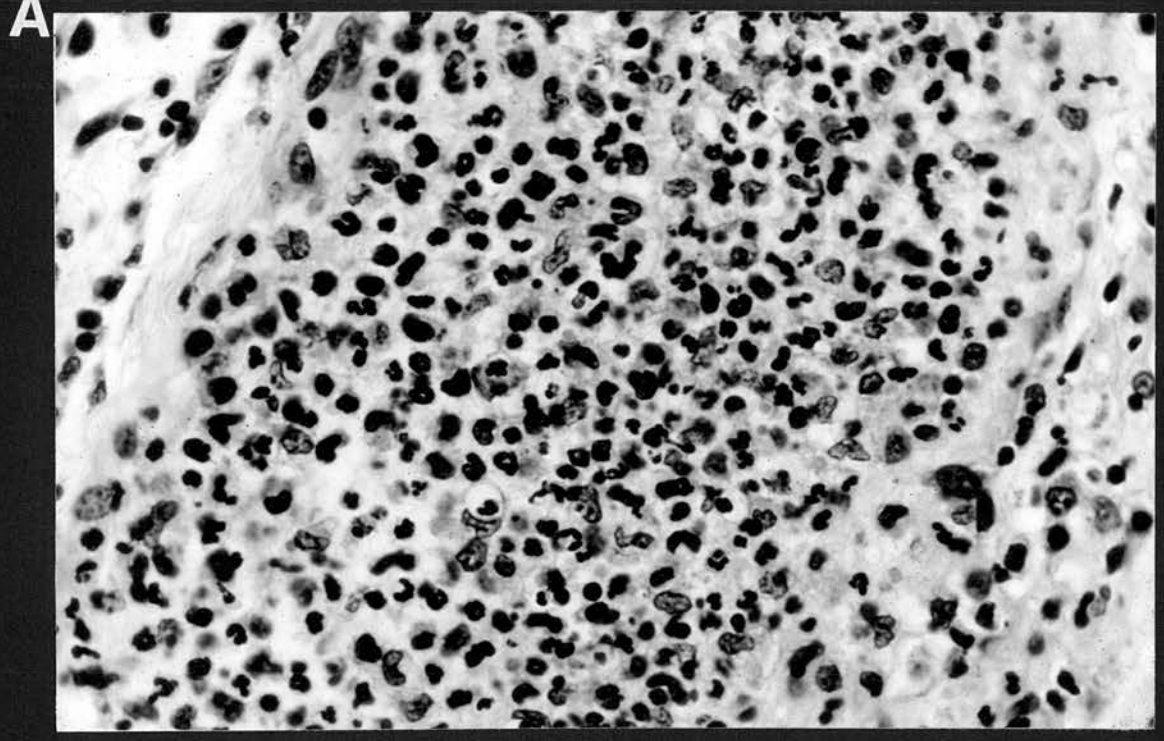


FIGURE 4.5

(A) Histological section of ovine skin seven days after infection. The cellular infiltrate consists mainly of mononuclear and polymorphonuclear cells (H + E x600).

(B) Histological section of ovine skin 10 days after infection. The cellular infiltrate consists predominantly of mononuclear cells, mainly large lymphocytes. (H + E x600).


A

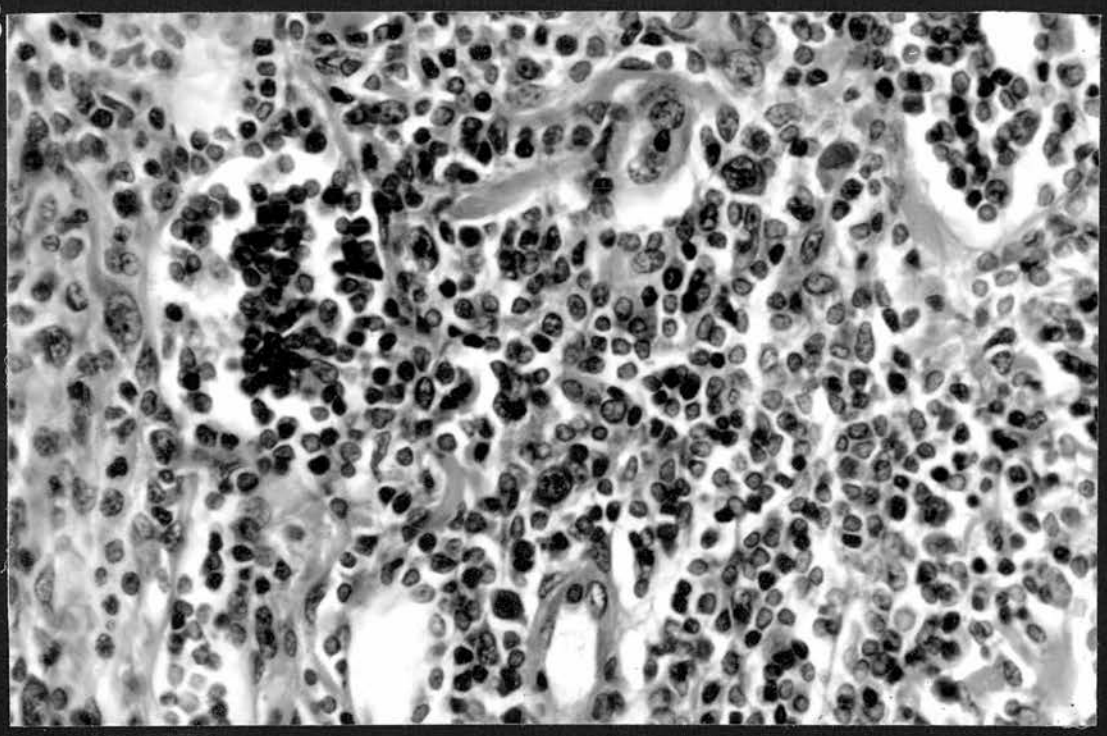

B

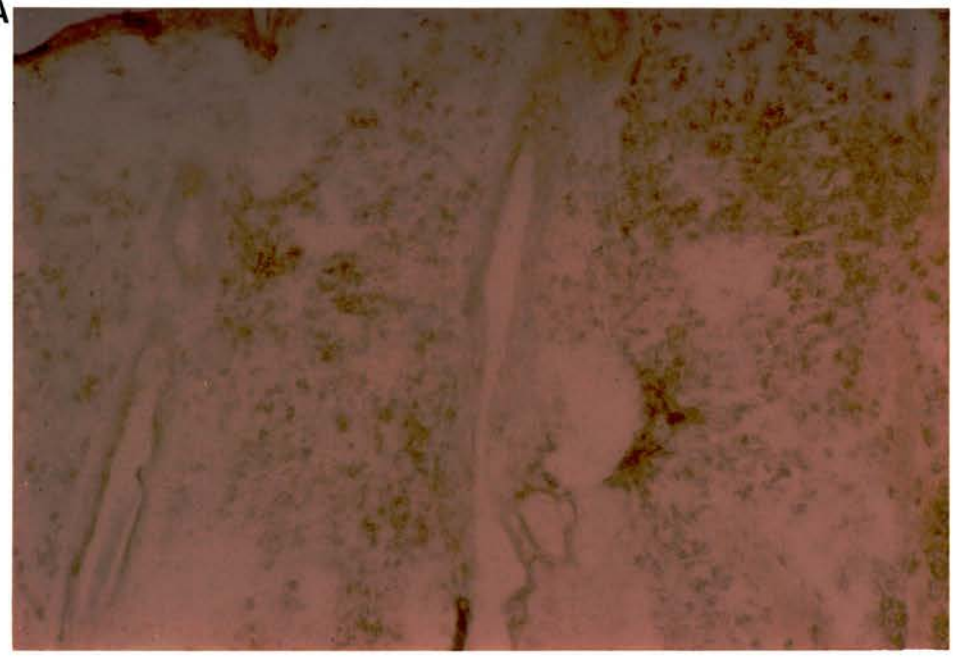
FIGURE 4.6 Cellular phenotypes in a local skin reaction of sheep seven days after infection with *T. congolense* TREU 1457. Immunoperoxidase staining with MAbs specific for ovine leucocyte subsets.

(A) $CD5^+$ cells diffusely distributed in the local skin reaction (x125).

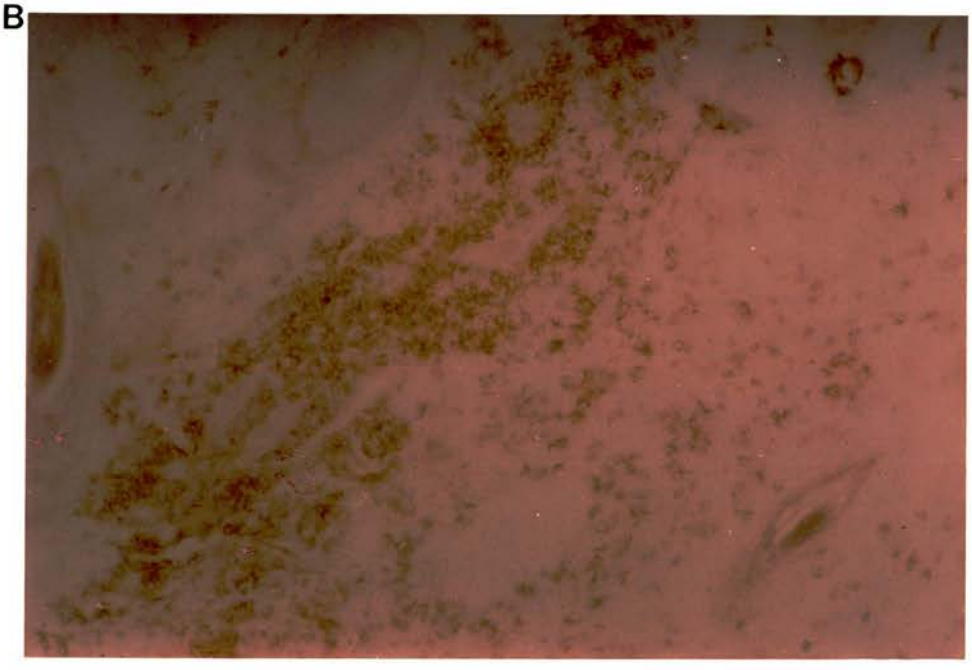
(B) $CD4^+$ cells in the local skin reaction are diffusely distributed although some focal perivascular aggregations are evident. These cells were the most predominant T lymphocytes seven days after infection (X125).

(C) $CD8^+$ cells in the local skin reaction. Few cells which are diffusely distributed. These cells were predominant from 10 days after infection (x125).

A



B



C

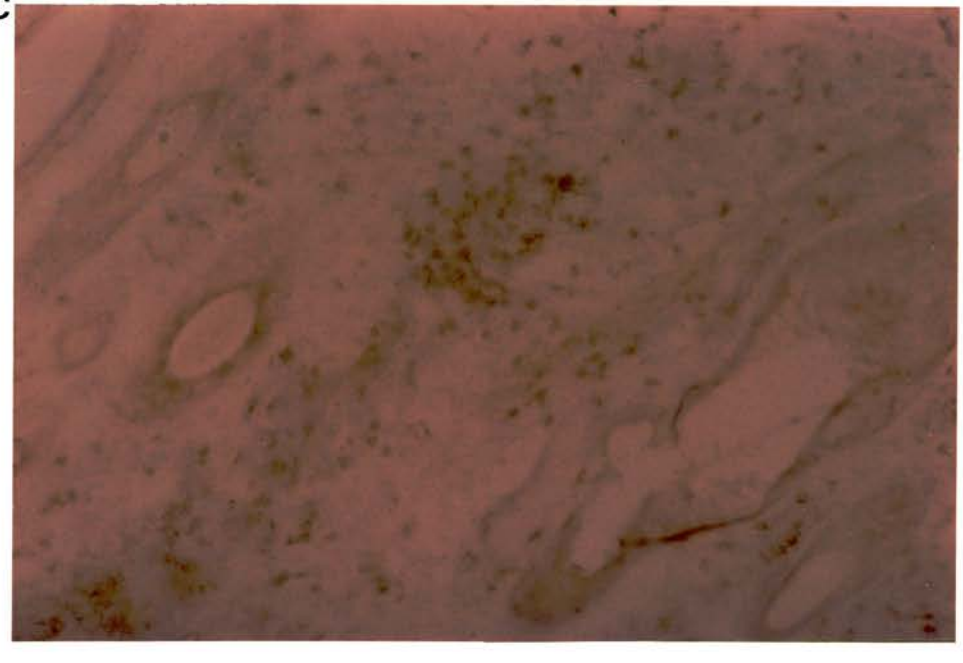


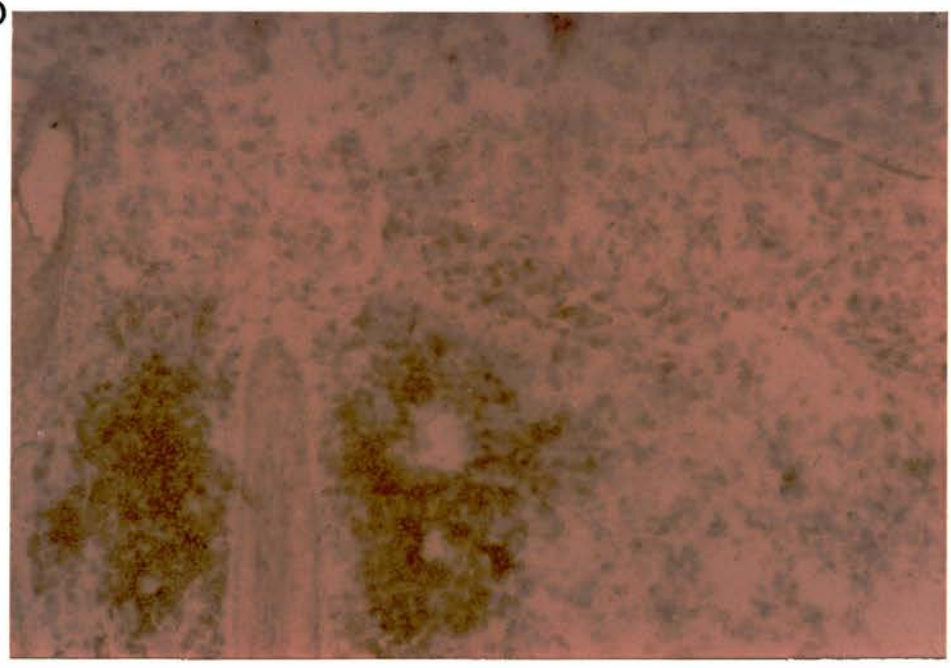
FIGURE 4.6 Cellular phenotypes in a local skin reaction of sheep seven days after infection with *T. congolense* TREU 1457. Immunoperoxidase staining with MAbs specific for ovine leucocyte subsets.

(D) $CD45R^+$ cells in the local skin reaction. These cells were distributed throughout the lesion but focal aggregations were also evident during this period (x125).

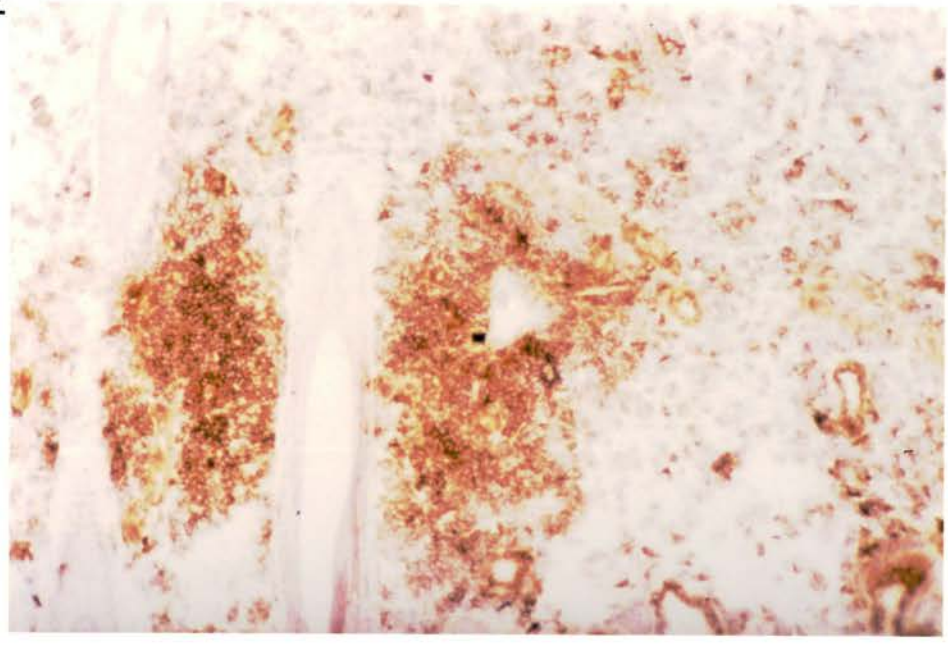
(E) MHC Class II^+ cells in the local skin reaction. These cells were present in the diffuse cellular infiltrate as well as in the $CD45R^+$ cell focal aggregates. Endothelial cells were also MHC Class II^+ (x125).

(F) Macrophages in the local skin reaction distributed throughout the lesion (x125).

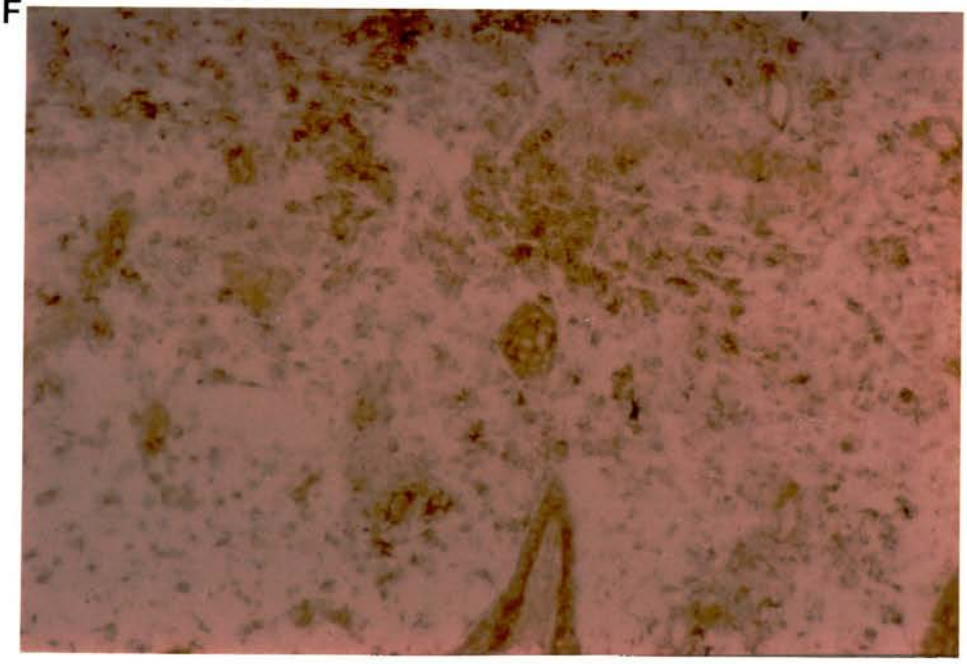
D



E



F



Normal skin contained only a few lymphocytes which were mainly $CD5^+$, $CD4^+$. $CD8^+$, with few $CD45R^+$ cells and macrophages expressing FcR. The ratio of CD4/CD8 in normal uninfected skin was found to be approximately one. There were increases in numbers of both $CD5^+$ and $CD45R^+$ cells between five to seven days after infection. $CD45R^+$ lymphocytes, which also expressed MHC Class II, were found in aggregates. Cells in the diffuse infiltrate reached their peak numbers between days five to seven p.i. when their ratio to the number of T cells was 1:1. Their numbers however declined thereafter but were still about ten times the preinfection values 26 to 30 days p.i.

Within the T cell population, there were parallel increases in $CD4^+$, $CD8^+$ cells which reached peak numbers five to 15 days after infection. The number of these cells increased up to 20 times pre-infection values. Five to seven days post-infection, there were more $CD4^+$ cells than $CD8^+$ cells and the CD4/CD8 ratio increased to 2.07. Thereafter, from 10 to 30 days p.i. the ratio declined and was less than one from 17 to 30 days as more $CD8^+$ were present than $CD4^+$ cells. The number of SBU-$T19^+$ cells ($\tau\delta$T cells) in the skin increased only about three-fold. The total number of cells expressing $CD4^+$, $CD8^+$ and SBU-$T19^+$ cells was greater than that of those expressing $CD5^+$ cells.

Although MHC Class II^+ cells and FcR^+ macrophages in the local skin reaction could not be easily quantified, there was immunohistological evidence of a marked increase in expression of MHC Class II (SBU-II) five to 15 days after infection. FcR was expressed on macrophages in the dermis and epidermis between 10 to 23 days. Some of the positive cells in the dermis and epidermis had dendritic-like processes. Fc receptor expression on B cells was observed only in the early stages of development of the skin reaction and diminished by 15 days after infection.

4.3.3 Effects of trypanocidal drug treatment on development and cellular phenotypes in skin reactions

The skin thickness of infection sites of sheep which had been treated with Berenil five days after infection, reached a peak of 5.2 mm on day six and decreased

to below 4 mm by day eight post-infection (Figure 4.3). Histologically, the magnitude of cellular infiltration in skin reactions from these sheep was diminished compared to that of untreated sheep.

The numbers of $CD5^+$, $CD4^+$, $CD8^+$ and B cells in inoculation sites of these sheep at 12 days after infection were lower than those of untreated, infected sheep at the same time (Table 4.2). All the cellular phenotypes especially B cells, decreased in number more rapidly from 17 days after infection compared with untreated, infected sheep. However, even by 30 days after infection (25 days after therapy) there was still more T cells ($CD5^+$, $CD4^+$ and $CD8^+$ cells) in the inoculation site than in control uninfected skin. By 26 days after infection there were hardly any B cells in the skin sections.

When the drug treated sheep were challenged with an heterologous serodeme of *T. congolense*, local skin reactions similar to those found in primary infections developed by seven days post-challenge. In contrast, no reactions occurred at sites where an homologous serodeme was inoculated. Histologically, cellular changes in the skin at sites of challenge with heterologous serodemes were similar to those seen in primary infections seven days after infection. These skin reactions, were characterized by extensive tissue oedema, disruption of collagen and marked cellular infiltrate containing mainly lymphocytes, lymphoblasts and a few macrophages, neutrophils and eosinophils. There was little evidence of cellular changes at sites in which metacyclic trypanosomes of the homologous serodeme had been injected. Seven days after inoculation skin sections from these sites histologically resembled normal, uninfected skin and contained few leucocytes with no detectable tissue oedema or disruption of collagen.

Skin sections from inoculation sites of treated sheep obtained seven days after challenge with the homologous serodeme (TREU 1885) contained low numbers of $CD5^+$, $CD4^+$, $CD8^+$ and B cells (Table 4.3) similar to uninfected skin. A site of inoculation with heterologous *T. congolense* serodemes (TREU 1457 or 1881) the

TABLE 4.2 **Median values (and ranges) of lymphocyte phenotypes in the skin reactions of sheep infected with *T. congolense* and then treated with Berenil five days after infection.**

Lymphocyte phenotype	Days after infection 12	17	26	30
CD5	50.5*(14-59)	54.0(27-88)	11.0(10-11)	41.5(12-60)
CD4	57.0(8-102)	38.5(24-50)	5.0(3-16)	13.0(10-39)
CD8	51.0(10-85)	51.5(33-68)	15.0(7-35)	7.0(4-21)
SBU-T19	5.0(0-30)	15.0(1-23)	1.0(0-2)	6.0(3-8)
CD45R	15.5(4-27)	7.0(0-13)	0(0-1)	0(0)
CD4/CD8	1.1	0.7	0.3	1.8

*Cells per 0.08 mm^2 field (x40 objective, x10 eyepiece)

TABLE 4.3 Median values (and ranges) of lymphocyte phenotypes in the day seven skin reaction of sheep infected with *T. congolense* (TREU 1885) treated with trypanocidal drug and rechallenged with homologous/heterologous serodemes.

Lymphocyte phenotype	*T. congolense* serodeme challenge TREU 1885	TREU 1881	TREU 1457
CD5	3.5*(2-15)	96.0(66-162)	181.0(121-239)
CD4	4.0(1-15)	67.5(43-139)	109.0(67-188)
CD8	10.0(3-15)	76.5(48-122)	120.0(79-129)
SBU-T19	0(6-1)	8.0(2-23)	5.0(2-14)
CD45R	0(0-6)	64.0(32-31)	120.5(93-177)
CD4/CD8	0.4	0.9	0.9

*Cells per 0.08 mm^2 (x40 objective, x10 eyepiece)

numbers of $CD5^+$, $CD4^+$, $CD8^+$, SBU-$T19^+$ and B cells were similar to those in skin reactions which developed following primary infection. There were more T cells ($CD5^+$) than B cells ($CD45R^+$) in these lesions compared with those from primary infections. The number of $CD4^+$. $CD8^+$ and $CD45R^+$ cells was roughly similar to those in primary infections with only a marginal increase in SBU-$T19^+$ cells. Infection sites with *T. congolense* TREU 1457 showed a higher cellular infiltration than those of *T. congolense* TREU 1881.

4.3.4 Effect of concurrent trypanosome infections on cellular phenotype dynamics

The two sheep infected with *T. congolense* TREU 1885 and then superinfected with either an homologous or heterologous serodemes failed to develop any macroscopically detectable skin reactions. Little evidence of cellular infiltration was observed in the skin of infected sheep seven days after they were superinfected with an homologous serodeme. However, following heterologous challenge, inoculation sites from one of the sheep showed some leucocyte infiltration which was low compared with that observed following primary infections.

In one of the sheep, skin at sites of inoculation with the heterologous serodeme showed a higher density of $CD5^+$, $CD4^+$ and $CD8^+$ cells than skin at sites which were inoculated with the homologous serodeme, but much lower than was found in the skin of sheep following a primary infection with *T. congolense*. The ratio of CD4/CD8 was roughly one. In the other sheep, only at sites inoculated with *T. congolense* TREU 1457 was there any infiltraton with low numbers of $CD5^+$, $CD4^+$, $CD8^+$ and $CD45R^+$ cells (Table 4.4)

4.4 Discussion

The initial host response to metacyclic forms of *T. congolense* is evident as early as three to five days after infection as illustrated by presence of degenerating trypanosomes in the skin. This response is unsuccessful in abrogating the establishment of infection in susceptible animals as trypanosomes proliferate and develop in large numbers in the dermal collagen, eliciting a localized skin reaction

TABLE 4.4 Median values (and ranges) of lymphocyte phenotypes in the skin reactions of sheep infected with *T. congolense* TREU 1885 and superinfected with homologous/heterologous serodemes.

Lymphocyte phenotype	Sheep 885 TREU 1885	1881	1457	Sheep 889 TREU 1885	1881	1457
CD5	8*(5-9)	50(36-62)	52(36-57)	4(1-5)	2(2-4)	25(18-57)
CD4	5(3-9)	42(30-50)	34.5(19-40)	3(2-7)	2(2-4)	17(6-33)
CD8	13(14-16)	42(30-58)	43(32-58)	6(2-9)	4(3-5)	12(8-33)
SBU-T19	1.5(1-2)	0	0(6-3)	0(0)	3.5(2-5)	4(3-9)
CD45R	1.5(1-2)	1.5(1-2)	0(0-3)	0(0)	1.0(0-4)	8(5-14)
CD4/CD8	0.4	1.0	0.8	0.5	0.5	1.4

*Cells per 0.08 mm^2 field (x40 objective, x10 eyepiece)

(Gray and Luckins, 1980; Emery and Moloo, 1981; Akol and Murray, 1982). The mechanisms which trigger the development of this reaction are still obscure. In this study there was evidence of mast cell changes usually associated with degranulation and release of chemical mediators implicated in a wide spectrum of inflammatory and immunological processes (Metcalfe, Kaliner and Doulon, 1981). Mast cell degranulation occurs in DTH reactions (Dvorak, Mihm and Dvorak, 1976; Askenase, 1980) and bullous pemphigoid (Dvorak *et al.*, 1982). Mast cell changes and granule release in DTH reactions are known to be induced by complement fragments or T cell derived factors (Thueson *et al.*, 1979; Askenase, Rosenstein and Ptak, 1983). Trypanosomes are known to activate the complement cascade through both the alternative and classical pathways (Malu and Tabel, 1986) which together with immune complexes likely be found at the inoculation site, might contribute to mast cell triggering with subsequent development of local skin reactions. Mast cells of man and large domestic animals are stimulated to release granules containing mediators such as histamine which cause marked erythema and increased vascular permeability and it is possible that histamine is one of the main mediators for development of these lesions in mammalian hosts.

The local skin reaction presents initially as an intense inflammatory response. While the histological features reported in this study resemble those described by other workers (Gray and Luckins, 1980; Emery and Moloo, 1981; Akol and Murray, 1982), the dissection of the leucocyte phenotype dynamics gives some insight into the cellular basis of this early host response. T cells are crucial in induction of immune responses directed against various infectious agents and in the development of immunopathological lesions. Healing of cutaneous lesions of *Leishmania mexicana amazonensis* infection in resistant C57BL/6 mice is associated with a local influx of both T helper cells ($L3T4^+$) and T suppressor/cytotoxic cells ($Lyt2^+$) into the dermis. In contrast, BALB/C mice which are highly susceptible to this infection develop non-healing lesions with minimal influx of T cells (McElrath *et al.*, 1987). T cells

predominate in the cellular infiltrate which occurs in collagen-II induced arthritis in rats (Klareskog *et al.*, 1983). Thymectomized rats do not develop such arthritic lesions underlining the role of T cells in pathogenesis of this condition. In *T. congolense* infected sheep, the cellular infiltrate observed in the local skin reactions is predominantly comprised of T cells. It is therefore possible that these cells are involved both in the induction of protective immune responses as well as pathogenesis of local skin reactions probably by elaborating an array of lymphokines.

During the early phase of the reaction, the majority of T lymphocytes belonged to the $CD4^+$ helper subpopulations but later, as the reaction receded most of the cells were of $CD8^+$ suppressor/cytotoxic phenotype. In the first phase of the response in the skin, the diffuse distribution of these T cells suggests that they were passively recruited into the inoculation site. However, their persistence in the lesion until 30 days post-infection indicated that they were either preferentially being recruited or that their migration into afferent lymph was selectively impeded. SBU-$T19^+$ cell subpopulation was only present in the lesion in low proportions throughout the course of skin reaction even though they make up about 15% of all peripheral blood lymphocytes. This phenotype, therefore does not appear to have any significant role in trypanosome induced local skin reactions. Similar observations have been made in gastric mucosa of sheep infected with *Haemonchus contortus* (Gorrell *et al.*, 1988a) and in inflammatory responses in the sheep liver to *Taenia hydatigena* infection (Meeusen *et al.*, 1989).

Observations that the total numbers of $CD4^+$, $CD8^+$ and SBU-$T19^+$ cells was greater than those of cells expressing $CD5^+$ is similar to findings from studies of lymphocyte subpopulations in gastric and intestinal mucosal lesions of sheep infected with *H. contortus* and *Trichostrongylus colubriformis* respectively (Gorrell *et al.*, 1988a, 1988b). It has been suggested that $CD8^+$ cells in non-lymphoid tissues lack $CD5^+$ antigens (Gorrell *et al.*, 1988a, 1988b). Following *in vivo* antigen stimulation of lymph nodes, activated peripheral T cells in efferent lymph of sheep lose their CD5

antigen expression while retaining the CD4 and CD8 antigens (Hopkins and Dutia, 1989). It is possible therefore that the changes seen in the skin are due to activation of T cells, although this would need to be confirmed by using double colour staining.

The distribution and presence of the various T cell subpopulations in inflammatory and immunological reactions might have a bearing on the outcome of infection. Effective protective immune responses in resistant hosts are characterized by development of granulomas which contain T lymphocytes, the majority of which are of $CD4^+$ phenotype. Tuberculoid leprosy in man (Modlin *et al.*, 1983; Narayanan *et al.*, 1983), listeriosis in mice (Naher *et al.*, 1985) and *T. hydatigena* in the liver of resistant sheep (Meeusen *et al.*, 1989) are all characterized by granulomatous lesions which are essentially protective immune responses. In contrast, granulomatous lesions in lepromatous leprosy (Modlin *et al.*, 1983; Narayanan *et al.*, 1983) and rhinoscleroma in man (Modlin *et al.*, 1983) represent host responses which are ineffective in eliminating invasive organisms. These lesions contain an admixture of $CD4^+$ and $CD8^+$ cells with a ratio of less than one. The early phase of local skin reactions in *T. congolense* infected sheep when the lesion is grossly visible and palpable (five to 15 days after infection) resembles an effective protective immune response as it contains a high proportion of $CD4^+$ cells. The later phase, (17 to 30 days after infection) when the reaction contains a higher proportion of $CD8^+$ cells thus resembling ineffective immune responses. However, in some other diseases, the proportion of $CD4^+$ and $CD8^+$ cells in lesions does not clearly indicate the effectiveness of immune responses. Healing and non-healing lesions of localized and diffuse cutaneous leishmaniasis are both characterized by a cellular infiltrate of T helper and T suppressor/cytotoxic cells in a ratio of less than one (Modlin *et al.*, 1985). The immunological effectiveness of these granulomas appeared to be related less to numbers and location of cells than to functional aspects of the cells and ability to generate lymphokines within the lesion (Modlin *et al.*, 1985). In humans, rats and mice, $CD4^+$ T cells consist of two functionally distinct subpopulations (Bottomly,

1988; Powrie and Mason, 1988). Inflammatory $CD4^+$ T cells (T_H1) secrete IL-2, gamma interferon and lymphotoxin and are required for effective immunity to intracellular pathogens such as *Leishmania.* Helper $CD4^+$ T cells (T_H2) provide help for specific antibody production, secrete IL-4 and IL-5 and are essential for effective humoral immune responses to extracellular pathogens. The effectiveness of a local immune response is therefore dependent on the type of $CD4^+$ T cell infiltrating the lesion. Hence, if a similar functional heterogeneity occurs in sheep $CD4^+$ T cells, changes in the numbers of these subpopulations could be responsible for the differences in responses seen in susceptible and resistant animals infected with *T. congolense.*

The most striking changes in the cell population is the infiltration of B cells ($CD45R^+$ cells) into the trypanosome induced local skin reactions during the early stages of development (five to seven days). These cells were found in aggregates resembling lymphoid follicles suggestive of local proliferation and antibody production. Similar B cell distribution occurs in the parasitic infections where protective immunity involves antibodies such as *T. hydatigena* in the liver of sheep (Meeusen *et al.*, 1989). Immunity to trypanosomes involves specific antibody production (Masake *et al.*, 1983; Black *et al.*, 1986; Mahan *et al.*, 1986) and is effective against metacyclic populations (Akol and Murray, 1985; Luckins *et al.*, 1990). A strong correlation also exists between the presence of antibodies and killing and phagocytosis of trypanosomes by macrophages (Lumsden and Herbert, 1967; Takayanagi *et al.*, 1974a,b; Stevens and Moulton, 1978). The presence of lysed trypanosomes in the skin reaction suggests that B cells proliferate and differentiate to plasma cells which produce specific antibodies locally. Many skin disorders such as canine dermatophilosis are associated with increased presence of immunoglobulins in the skin (Moore *et al.*, 1987). Attempts to demonstrate the presence of anti-trypanosomal antibodies in local skin reactions have been unsuccessful and

extravascular tissue fluid collected from around skin reactions in rabbits contain only low levels of antibodies (Silayo, 1984).

MHC Class II^+ cells comprised a sizeable proportion of cells infiltrating local skin reactions. These cells were mainly B cells which express MHC Class II antigens constitutively (Ellis *et al.*, 1987; Emery, MacHugh and Ellis, 1987) and as judged by the identical distribution within the skin reaction. However, MHC Class II is also expressed on activated T cells and macrophages/dendritic cell populations (Singer and Hodes, 1983; Puri, Mackay and Brandon, 1988). Induction of MHC Class II expression on macrophages and other cells is mediated, at least in part, by gamma interferon (IFN-y) produced by activated lesions (Kaye, 1987). This might be the case in trypanosomal skin reactions. MHC Class II^+ cells are known to be potent antigen presenting cells and regulators of immune responses, especially in the augmentation of T-helper cell function (Armstrong *et al.*, 1987). The mixture of these cells with T lymphocytes in the skin reaction might enhance the development and induction of an effective early protective immune response.

Fc receptor expression observed early in the skin reactions is probably a result of the presence of high numbers of B cells since this expression diminished rapidly with the decrease in B cells seven days after infection. Fc receptors on B cells play a role as immune regulators (Lydyard and Fanger, 1982) and could be essential in antigen presentation to T cells within the local skin reaction, or internalization and processing of trypanosomal antigens. Fc receptor expression on macrophages is important in enhancing opsonic adherence, phagocytosis and degradation of immune complexes (Male, Champion and Cooke, 1987). FcR^+ macrophages were present during the peak and regression of the local skin reaction and might be responsible for the resultant augmentation of phagocytosis and destruction of opsonized trypanosomes.

The size of the local skin reaction is dependent on the number of metacyclic trypanosomes inoculated into the skin of the susceptible host (Dwinger *et al.*, 1987b). The reduction of the number of viable trypanosomes at inoculation sites by

trypanocidal therapy of infected sheep resulted in the reduction in size of the skin reaction, the degree of cellular infiltration and the numbers of different lymphocyte subpopulations, especially B cells. These sheep developed characteristic skin reactions seven days after challenge with heterologous but not with homologous *T. congolense* serodemes. These sheep appeared to be immune to homologous challenge. However, since trypanosome infected ruminants treated before 15 days after infection are fully susceptible to homologous challenge (Emery *et al.*, 1980; Akol and Murray, 1983), it is possible that the development of the skin reaction was only delayed in onset.

Active infections in sheep prevented the development of skin reactions following both homologous and heterologous challenge. This finding concurs with studies by other workers (Luckins and Gray, 1983; Dwinger *et al.*, 1989) which have demonstrated that existing trypanosomal infections interfere with establishment of superinfections. Although no gross skin reactions developed following heterologous challenge, a mononuclear cell infiltrate comprising mainly T cells was observed. The mechanisms operating in interference of establishment of superinfections are not clear. Trypanosome infections result in generation of antigen non-specific suppressor T cells or macrophages at least in mice (Jayawardena and Waksman, 1977; Yamamoto *et al.*, 1985) which might be responsible for suppression of development of immunological reactions. Trypanosome infections in cattle are also accompanied by a decline in T cell subpopulations and responsiveness to specific antigens (Ellis *et al.*, 1987; Emery *et al.*, 1980c). Alternatively, inflammatory responses during the course of the disease could result in generation of anti-inflammatory factors which might be responsible for the depression of local skin reactions.

In conclusion, marked cellular infiltration and changes in T cell subpopulations ($CD5^+$, $CD4^+$ and $CD8^+$) and B cells occur in *T. congolense* induced local skin reactions in sheep. These changes might lead to enhanced responsiveness to metacyclic trypanosomes but are not effective in preventing the establishment of

infection. However, it is possible that qualitative and quantitative differences in cellular phenotypes in the skin may be involved in determining the outcome of infection. It has already been demonstrated that only small local skin reactions develop in trypanotolerant N'dama cattle and wild animals such as African buffalo (Dwinger, 1985; Akol *et al.*, 1986a). Studies in cellular phenotypes in infected skin of such animals may indicate the role of different lymphocyte subpopulations in this resistance to infection.

CHAPTER FIVE

CELLULAR PHENOTYPES IN *TRYPANOSOMA CONGOLENSE* INFECTED SHEEP: IMMUNOHISTOLOGY OF LYMPH NODES DRAINING LOCAL SKIN REACTIONS

5.1 Introduction

Generalized lymph node enlargement is a characteristic feature of trypanosome infections in both domestic and laboratory animals (Fiennes, 1970; Losos and Ikede, 1972; Murray *et al.*, 1974; Moulton and Coleman, 1977; Murray *et al.*, 1980; Valli and Forsberg, 1980). Histological evidence reveals that this lymphoid enlargement is due to marked proliferative activity which occurs in B cell dependent follicular areas with subsequent development of germinal centres (Murray *et al.*, 1974a; Mansfield, 1978; Morrison and Murray, 1979). At the same time T cell dependent areas are depleted of small lymphocytes and are infiltrated by plasma cells and macrophages (Murray *et al.*, 1974; Mansfield and Bagasra, 1978). However, in longstanding infections in laboratory animals and cattle, lymph nodes are atrophic and hardened, due to protracted cellular depletion (Fiennes, 1970; Kaliner, 1974; Morrison and Murray, 1979; Murray *et al.*, 1980).

Development of local skin reactions in mammals following bites by tsetse flies infected with *T. congolense* is associated with marked enlargement of regional draining peripheral lymph nodes similar to that observed in established infections (Luckins and Gray, 1979; Gray and Luckins, 1980; Akol and Murray, 1982). This lymph node enlargement is due to an intense proliferative lymphoid response characterized by formation of numerous follicles, large active germinal centres in the cortical areas, and an increase in the number of plasma cells in the medullary cords (Murray *et al.*, 1980). Migration of cells and trypanosomes from the skin reaction (Gray and Luckins, 1980; Akol and Murray, 1982; Akol and Murray, 1986) might contribute to the enlargement and induction of a considerable lymphoproliferative response in the draining lymph node and therefore contribute to an increased cellular output via the efferent lymphatic. Indeed, lymphatic cannulation studies in cattle and goats infected with *T. congolense* have demonstrated an increase in lymphocyte and lymphoblast output in the efferent lymph from the lymph node draining local skin reactions (Akol and Murray, 1986; Dwinger *et al.*, 1990). The significant role that the draining lymph node plays in the induction of the early immune response to cyclically

transmitted infections is also indicated by presence of trypanosome-specific antibodies in the efferent lymph of infected cattle, goats and sheep (Akol and Murray, 1986; Dwinger *et al.*, 1990; Luckins *et al.*, 1990).

The dynamics of cellular phenotypes during the development of local skin reactions indicated the occurrence of a localized inflammatory reaction whose cellular phenotype composition changed over time. The present experiment describes the changes in distribution and localization of cellular phenotypes within lymph nodes draining *T. congolense* induced local skin reactions in sheep.

5.2 Materials and Methods

5.2.1 Trypanosomes and infection

Sheep were infected with one or more of the three stocks of *T. congolense*; TREU 1457, TREU 1881 and TREU 1885. Infection was established by intradermal inoculation of culture-derived metacyclic forms into the area of skin drained by either prescapular or prefemoral lymph nodes.

5.2.2 Experimental design

Full details of the experimental design is outlined in Table 5.1. Ten sheep were employed in the study and lymph nodes were obtained on the days indicated. Two of the sheep were infected with two stocks, TREU 1881 and TREU 1457 simultaneously in areas of skin drained by prefemoral and prescapular lymph nodes respectively. To obtain the nodes, sheep were killed six to 30 days after infection. Lymph nodes were also collected from uninfected sheep for control samples. For immunohistochemistry, a piece of lymph node encompassing the whole cross-section of the tissue was snap frozen in dry ice and isopentane, wrapped in aluminium foil and then stored at -70°C. Cellular phenotypes were localized in frozen tissue sections by an indirect immunoperoxidase staining as described in Section 3.9. Formalin fixed, paraffin embedded 5 μm thick lymph node sections were stained with haematoxylin and eosin or with Giemsa (Drury and Willington, 1980). All the sections were observed under light microscopy.

TABLE 5.1 Experimental design

Sheep	Breed	Serodeme of *T. congolense* used for infection	Infection sites	Days after infection when LNS were obtained LPF	RPF	LPS	RPS	Days after infection when sheep were killed
101	Suffolk	TREU 1457	3	0	6	10	13	13
102	Suffolk	TREU 1457	3	0	6	10	13	13
103	Blackface	TREU 1885	3	0	7	10	13	13
104	Blackface	TREU 1885	3	0	7	10	13	13
105	Blackface	TREU 1885	3	0	7	10	13	13
106	Blackface	TREU 1885	3	0	7	10	13	13
107	Suffolk	TREU 1885	4	15	15	15	15	15
108	Suffolk	TREU 1885	4	30	30	30	30	30
109	Suffolk	TREU 1881/	2	7	7	-	-	7
		TREU 1457	2	-	-	7	7	
110	Suffolk	TREU 1881/	2	7	7	-	-	7
		TREU 1457	2	-	-	7	7	

LNS - Lymph Nodes
LPF - Left prefemoral lymph node
LPS - Left prescapular lymph node
RPF - Right prefemoral lymph node
RPS - Right prescapular lymph node

5.3 Results

5.3.1 Histopathology

Normal lymph nodes showed a relatively low level of proliferative activity. These nodes contained a few primary follicles mainly in the outer cortex lacked germinal centres (Figure 5.1). The paracortical areas were densely populated with small lymphoid cells. Subcapsular, cortical trabecular and medullary sinuses contained few lymphocytes and macrophages.

Lymph nodes from infected sheep obtained six to 15 days post-infection (p.i.) showed varying degrees of lymphoid changes especially on days seven, 10 and 13 p.i. (Figure 5.3). Lymph node enlargement was associated with a marked increase in follicular hyperplasia. Numerous follicles containing germinal centres were observed in the nodes especially along trabecular sinuses. These follicles which contained large lymphocytes and macrophages were larger than those observed in control nodes and were found in paracortical and medullary areas where they seemed to displace medullary cords and sinuses. The paracortical areas were relatively reduced due to compression by the numerous follicles. Many macrophages were evident in these areas giving it a 'starry sky' or 'moth eaten' appearance (Figure 5.2). Subcapsular and cortical trabecular sinuses contained numerous macrophages, lymphocytes and lymphoblasts. Medullary cords contained an increased number of lymphocytes, lymphoblasts and plasma cells while the medullary sinuses was packed with macrophages, lymphocytes and lymphoblasts.

In lymph nodes obtained 30 days p.i. there was a decrease in follicular proliferative activity. Fewer follicles were present during this period and the sinuses contained fewer lymphocytes and macrophages. However, the paracortical areas still contained numerous macrophages. Medullary cords contained fewer plasma cells compared to nodes obtained during the peak of lymph node reactivity.

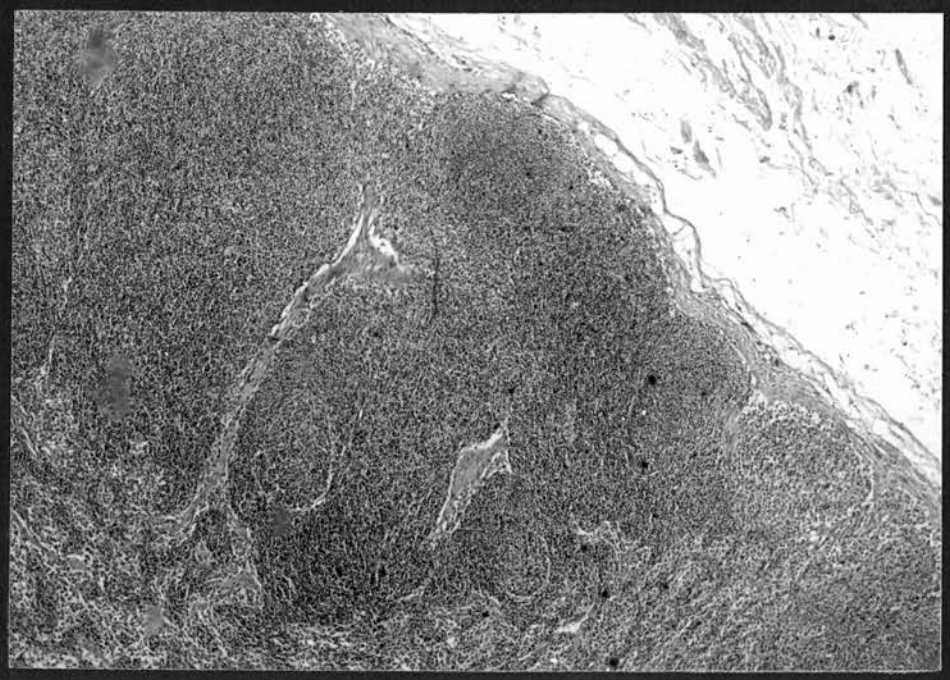
A large number of trypanosomes was observed in the afferent lymphatics entering lymph nodes obtained seven and 10 days p.i. (Figure 5.2). Trypanosomes

FIGURE 5.1 Histological section of a prefemoral lymph node from uninfected sheep. Few lymphoid follicles are present and the parafollicular areas are densely populated with lymphocytes (H + E x50).

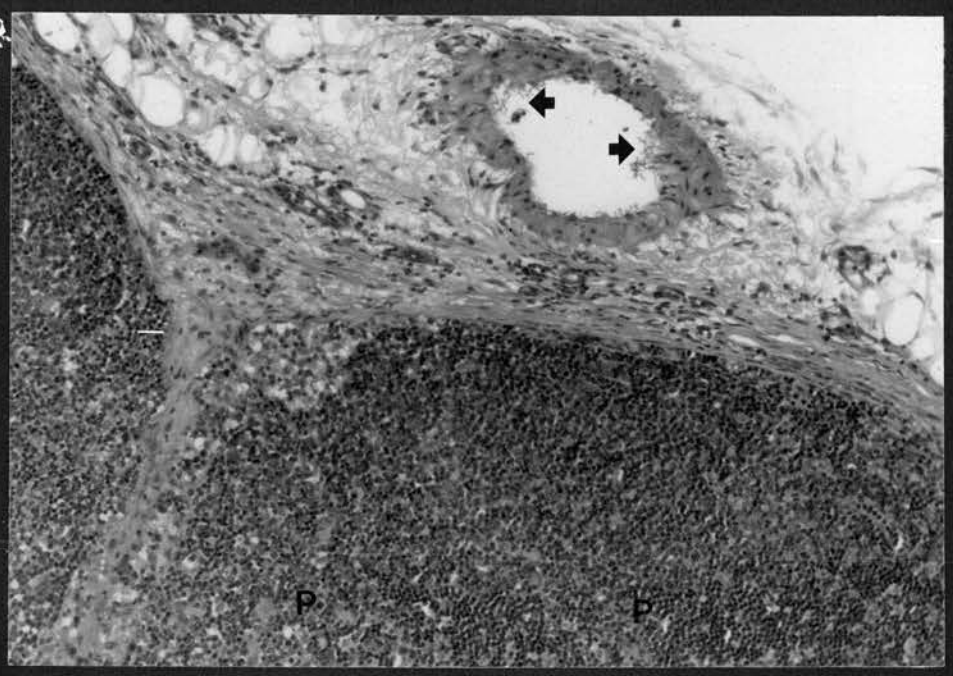
FIGURE 5.2 Histological section of prescapular lymph node draining local skin reaction in sheep seven days after intradermal inoculation with culture-derived metacyclic forms of *T. congolense* TREU 1457. Trypanosomes (arrowed) are present in the afferent lymphatic ducts. The paracortical (P) areas are sparsely populated with lymphocytes (H + E x125).

FIGURE 5.3 Histological section of prefemoral lymph node draining a local skin reaction in sheep 10 days after infection. Numerous follicles (F) are present in cortical, paracortical and medullary areas. The paracortical areas (P) are relatively reduced. M, Medulla; C, lymph node capsule (H + E x50).

5.1



5.2



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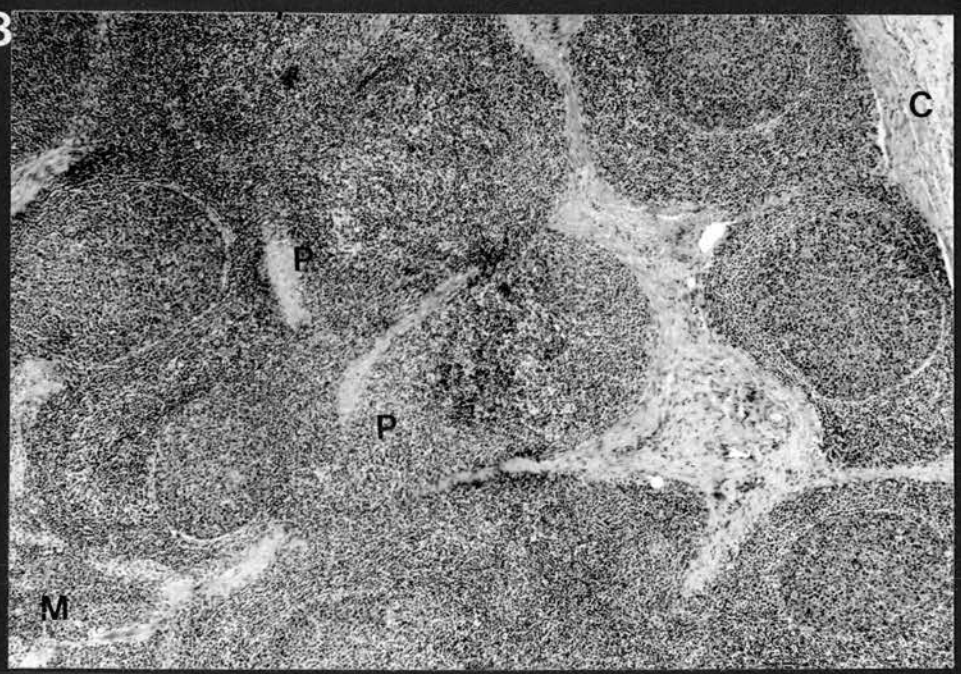


were also found in subcapsular and cortical trabecular sinuses but not in medullary sinuses or solid lymphoid tissues. No trypanosomes could be found in lymph nodes obtained 13, 15 or 30 days p.i.

5.3.2 Immunohistology of draining lymph nodes

5.3.2.1 B cells, MHC Class II and macrophage/dendritic cell phenotypes

Lymph nodes from control uninfected sheep contained only a few B cell follicles, which stained with CD45R$^+$ (Figure 5.4). Few CD45R$^+$ cells were present in the paracortical and medullary areas of these nodes. MHC Class II$^+$ cells were restricted to follicular areas where both lymphoid cells and follicular interdigitating cells (FDCs) expressed high levels of the antigen. In the paracortical areas, few MHC Class II$^+$ lymphoid cells were present although several intensely staining interdigitating cells (IDCs) with dendritic cell morphology were present. The medullary cords, cortical and medullary sinuses contained few MHC Class II$^+$ cells. CD1 antigen was present on IDCs and on low levels in B cells while IDCs, FDCs and few macrophages in the medulla expressed FcR.

Lymph nodes obtained six to 15 days p.i. showed an increase in population and distribution of CD45R$^+$ cells (B cells) and MHC Class II$^+$ cells. These changes were observed especially in lymph nodes obtained on days seven, 10 and 13 p.i. These nodes showed increased presence of CD45R$^+$ cells in the expanded follicular areas (Figure 5.4) which extended from the cortex into the medullary sinus and especially along the cortical trabecular sinuses. These cells were also increasingly present in paracortical areas and medullary cords.

The distribution and alteration in population of MHC Class II$^+$ cells was similar to that of CD45R$^+$ cells. Numerous MHC Class II$^+$ cells were located in B cell follicles, paracortex, and cortical trabecular sinuses and medullary sinuses (Figure 5.4). Most of the cells in sinuses and paracortex had the morphology of macrophages/dendritic cells. These cells were reactive to MAb VPM 32 (FcR receptors on macrophages) and had low levels of CD1 antigen. VPM32$^+$ cells packed

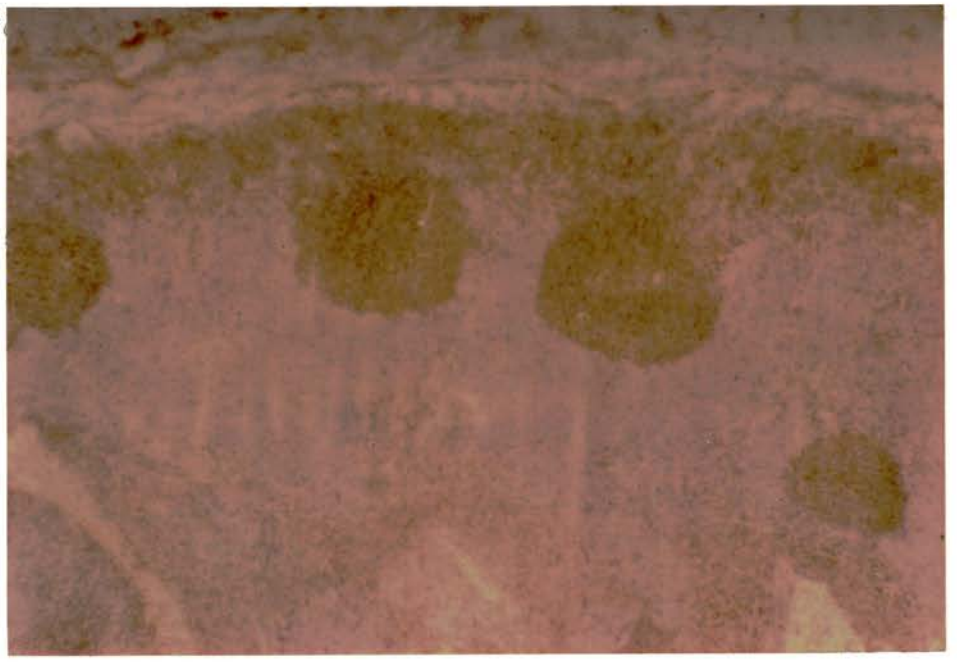
FIGURE 5.4 Immunoperoxidase staining of a cryostat section of a prefemoral lymph node draining local skin reaction in sheep seven days after intradermal infection with *T. congolense* TREU 1457.

(A) $CD45R^+$ staining of prefemoral lymph node draining uninfected skin. Few lymphoid follicles are present (x125).

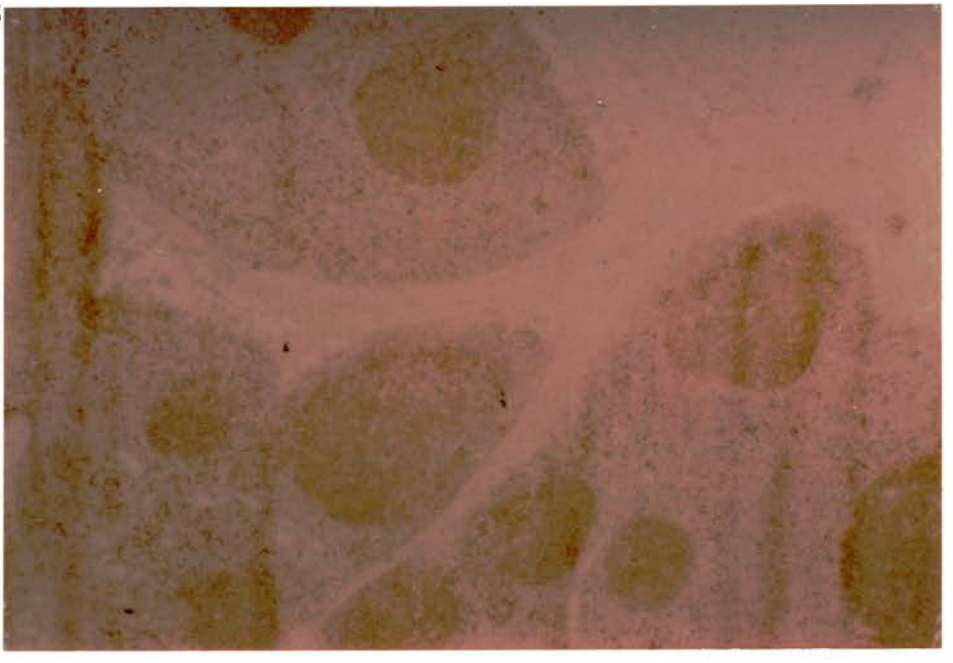
(B) $CD45R^+$ cells in the lymph node draining a local skin reaction in sheep seven days after infection. Numerous $CD45R^+$ follicles are present (x50).

(C) MHC Class II^+ cells in lymphoid follicles of a lymph node draining a local skin reaction seven days after infection (x50).

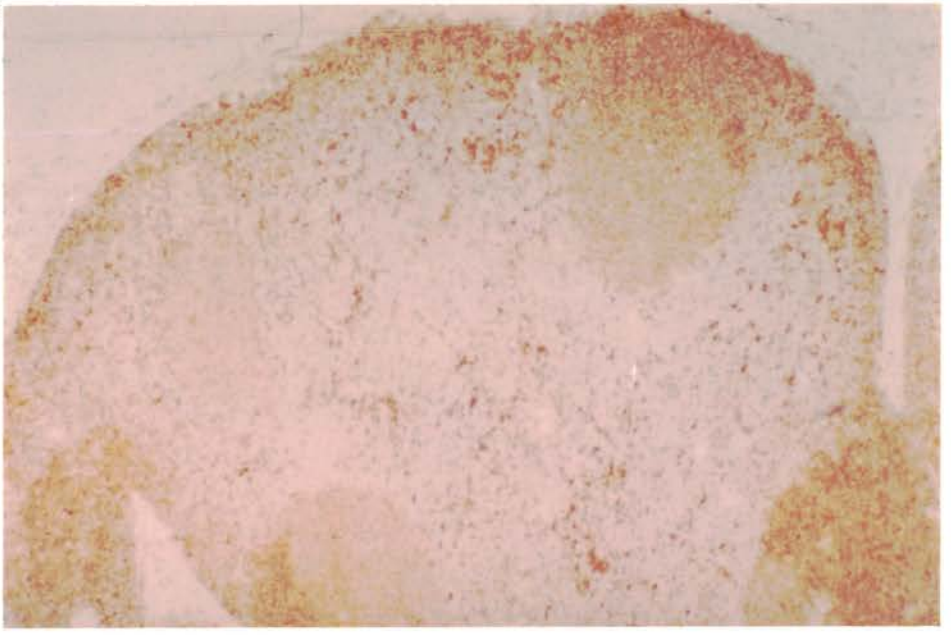
A



B



C



the medullary and cortical trabecular sinuses in lymph nodes obtained between days seven and 13 p.i. (Figure 5.5).

Lymph nodes obtained 30 days p.i. contained fewer B cell ($CD45R^+$ cells) follicles and few of these cells were present in paracortex, and medullary area compared with lymph nodes obtained six to 15 days p.i. The population of cells expressing MHC Class II antigens in cortical and medullary areas of these nodes was reduced. Similarly fewer FcR^+ macrophages were present in the FcR^+ medullary sinuses while still increasingly present in the paracortex.

5.3.2.2 T cell subsets

The paracortex of normal lymph nodes was densely populated with T cells ($CD5^+$ cells) the majority of which were $CD4^+$ together with some $CD8^+$ cells. B cell follicles contained few $CD5^+$ and $CD4^+$ cells. Similarly, the medullary cords contained scattered $CD5^+$ and $CD4^+$ cells but few $CD8^+$ cells. Cells expressing SBU-T19 ($\tau\delta$ T cells) were only found along subcapsular and trabecular sinuses.

Lymph nodes obtained from infected sheep six to 15 days p.i. showed marked reduction in T cell areas due to the presence of numerous expanding B cell follicles and expansion of the medullary regions (Figure 5.6). These changes were particularly evident in lymph nodes obtained seven to 13 days p.i. In comparison with control lymph nodes, the density of T cell subsets ($CD5^+$, $CD4^+$ and $CD8^+$ cells) in the paracortex were reduced. No major alterations were observed for $SBU\text{-}T19^+$ cells. Few $CD5^+$ and $CD4^+$ T cells were present in the numerous B cell follicles either in the cortex or medullary areas. However, these cells together with $CD8^+$ cells were part of the lymphoid mantle surrounding the follicles. In the medullary cords of lymph nodes from infected sheep, an increased presence of $CD8^+$ cells was observed from seven days p.i. (Figure 5.5).

Lymph nodes obtained 30 days p.i. still showed relatively reduced paracortical areas with sparse population of $CD5^+$, $CD4^+$ and $CD8^+$ cells compared to lymph

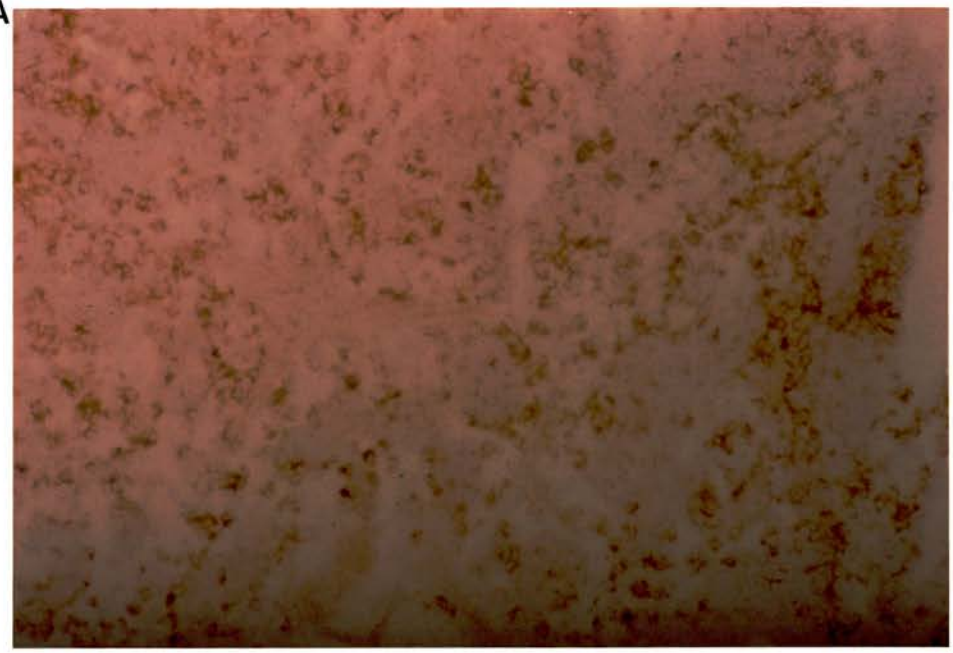
FIGURE 5.5 Immunoperoxidase staining of cryostat sections of prefemoral lymph nodes draining local skin reactions in sheep seven days after intradermal infection with *T. congolense* TREU 1457.

(A) Macrophages in the medulla of lymph node draining uninfected skin. Few macrophages ($VPM32^+$) are present in the medullary sinuses (x125).

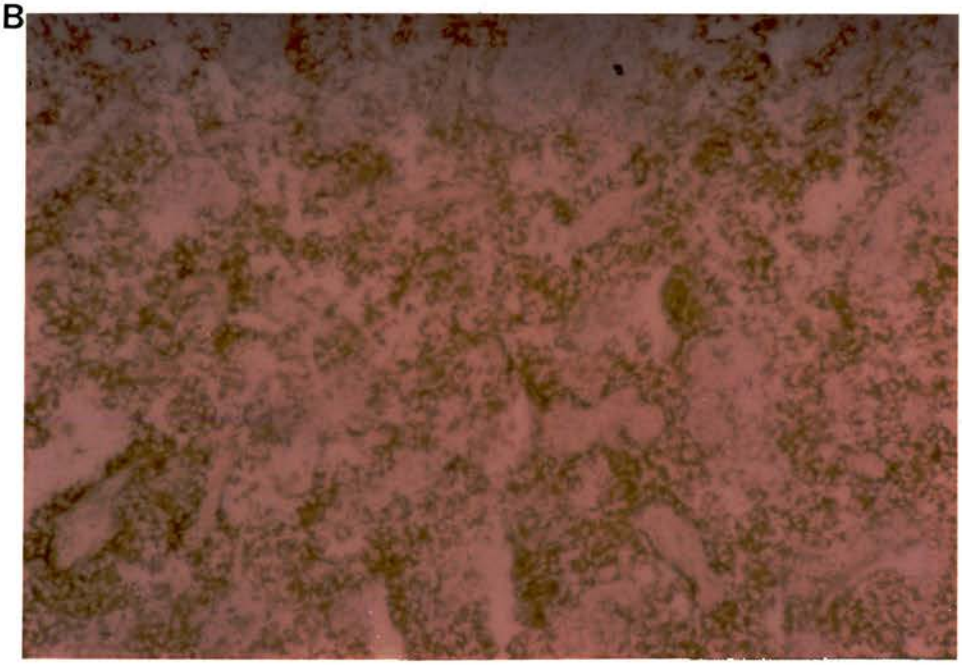
(B) Macrophages in medulla of prefemoral lymph node draining a local skin reaction in sheep seven days after infection. Numerous macrophages are present in the medullary sinuses (x125).

(C) $CD8^+$ cells in the medulla of prefemoral lymph node draining a local skin reaction in sheep seven days after infection. Numerous $CD8^+$ cells are present especially around follicles in the medullary cords (x50).

A



B



C

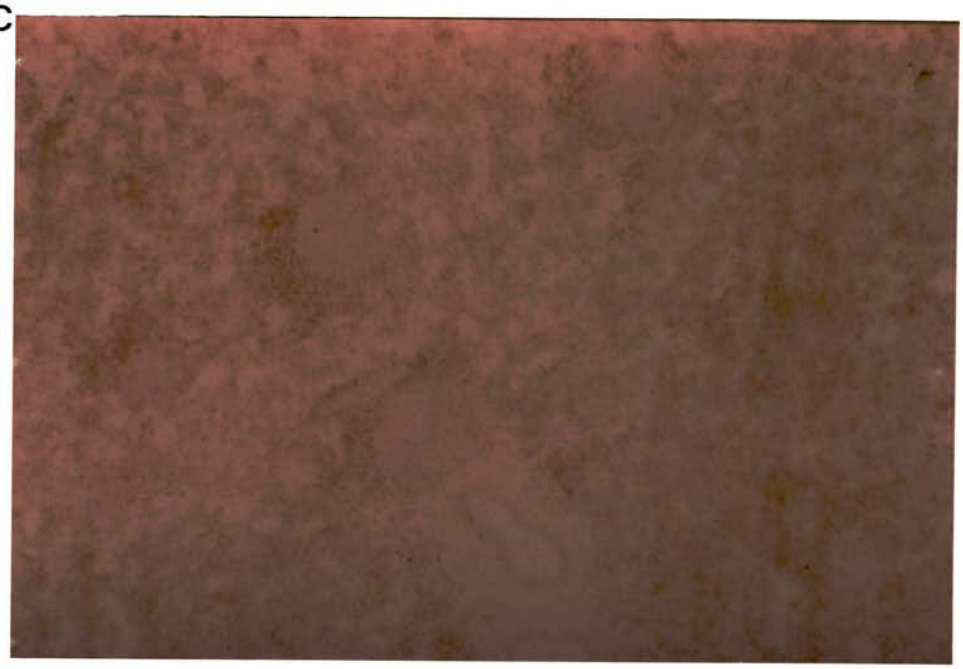


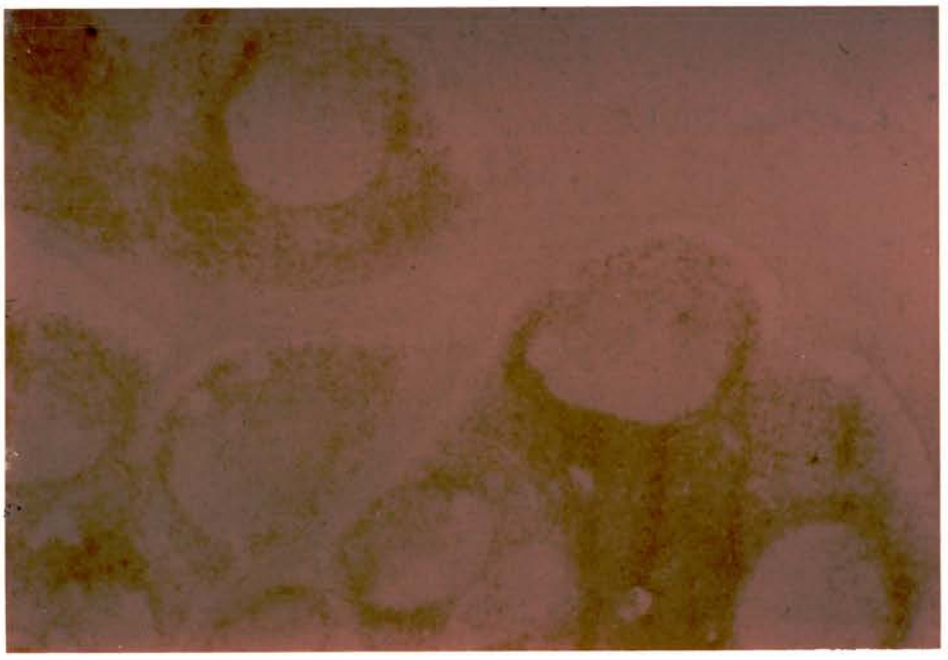
FIGURE 5.6 Immunoperoxidase staining of cryostat sections of prefemoral lymph node draining a local skin reaction in sheep seven days after intradermal infection with *T. congolense* TREU 1457.

(A) $CD5^+$ cells in the lymph node cortex. These cells are sparsely distributed in parafollicular areas. Few $CD5^+$ cells are present in the follicular areas (x50).

(B) $CD4^+$ cells in the lymph node cortex. These cells were more sparsely distributed in parafollicular areas than in normal lymph nodes. The follicles contain few $CD4^+$ cells (x125).

(C) $CD8^+$ cells in the lymph node cortex most of which are located in the parafollicular areas. Very few $CD8^+$ cells are present in follicular areas (x125).

A



B

C

nodes from control sheep. However, numerous $CD8^+$ cells were still present in the medullary cords.

5.4 Discussion

Generalized lymph node enlargement was observed in all sheep following intradermal inoculation of culture-derived metacyclic forms of *T. congolense.* Histologically, lymph node enlargement was characterized by marked follicular hyperplasia involving B cell areas, increased number of plasma cells in medullary areas, apparent reduction of the paracortical area and depletion of small lymphocytes. These changes were similar to those observed in generalized lymph node enlargement in established *T. congolense* infections in cattle and in laboratory rodents (Valli and Forsberg, 1979; Murray *et al.*, 1980; Morrison *et al.*, 1982c). The changes occurred at a time when trypanosomes, and increased number of cells were observed in afferent lymphatic vessels and subcapsular and cortical trabecular sinuses. The presence of trypanosomes in lymph nodes draining local skin reactions in cattle and sheep following cyclical *T. congolense* infection has been reported previously (Luckins and Gray, 1979). The migration of trypanosomes and cells from the local skin reaction therefore appears to be the stimulus for local lymph node enlargement.

Immunohistochemical techniques have revealed that lymph node hyperplasia is associated with an increase in the population of $CD45R^+$ cells and MHC Class II^+ cells. These cells were present in follicular aggregations in the cortex, paracortex and medullary cords. This immunohistological feature is highly suggestive of a strong antibody response occurring in the node during this period since such immune reactions are associated with proliferative activity of B cell areas (Morrison *et al.*, 1986). Marked B cell stimulation is also a feature of lymph nodes draining *Leishmania major* lesions in mice (Solbach *et al.*, 1987; Lohoff, Matzner and Röllinghoff, 1988) as well as in many other infectious diseases such as Chagas' disease, Malaria, Schistosomiasis and Leprosy (Freeman *et al.*, 1970; Turk and Bryceson, 1971; Greenwood and Vick, 1975; d'Imperio *et al.*, 1986). Proliferation

and accumulation of B cells in draining regional lymph nodes can be brought about in various ways. The distinct pattern of localization of B cell follicles along and adjacent to cortical trabecular sinuses suggests that this B cell stimulation is due to entry of lymphocytes and trypanosomes from the local skin reactions into the draining node (Morrison *et al.*, 1986). In *L. major* infections in mice, B cells from lesion draining lymph nodes are activated to undergo proliferation and differentiation by $L3T4^+$ (T helper) cells from the same node (Lohoff *et al.*, 1988). This does not seem to be the main mechanism of B cell stimulation in lymph nodes of trypanosome infected hosts. Trypanosome induced B cell proliferation has been demonstrated in T lymphocyte-depleted mice (Askonas *et al.*, 1979) and congenitally T cell-deficient nude mice (Clayton *et al.*, 1979; Kobayakawa *et al.*, 1979). A second hypothesis favoured by these observations is that trypanosomes invading the lymph node produce or induce production of a B lymphocyte mitogen (Urquhart *et al.*, 1973; Greenwood, 1974). Trypanosomal antigens have been shown to possess B cell mitogenic activity (Mansfield, Craig and Stelzer, 1976; Assoku and Tizard, 1978; Tizard *et al.*, 1978b; Clayton *et al.*, 1979). Alternatively, trypanosomes might induce host cells to produce B cell mitogens. It has been demonstrated that after phagocytosis of lethally irradiated trypanosomes, or trypanosomal membrane fractions, activated macrophages from uninfected mice are capable of stimulating non-specific B-lymphocyte proliferation (Grosskinsky and Askonas, 1981; Sacks *et al.*, 1982).

The increase in the number of MHC Class II^+ cells in lymph nodes was due to the expansion of the B cell population since the distribution of these two cell phenotypes was closely related. However, more MHC Class II^+ cells were present in paracortical areas of infected lymph than uninfected lymph nodes. Although some of these cells were B cells, or activated T cells, the majority were interdigitating cells (IDCs) or macrophages. These cells were also present in subcapsular and trabecular sinuses. Macrophages and dendritic or 'veiled' cells enter the lymph node through afferent lymph bringing along antigens from the skin (Hein, McClure and Miyasaka,

1987). These cells, which express high levels of MHC Class II antigen are important accessory cells in T cell-mediated immune responses (Van Voorhis *et al.*, 1983; Budjoso *et al.*, 1989). The presence of these cells together with B cells in paracortical areas of infected lymph nodes caused the apparent decrease in density of T cell subsets ($CD5^+$, $CD4^+$ and $CD8^+$ cells). This did not indicate a depletion of T cells since the numerous B cell follicles in cortex, paracortex and medulla were each surrounded by a mantle of $CD5^+$, $CD4^+$ and $CD8^+$ cells. Indeed, the admixture in the paracortex of T cells with activated macrophages/IDCs and B cells which are potent antigen presenting cells might result in antigen specific T cell populations capable of mediating B cell proliferation as observed in experimental murine leishmaniasis (Lohoff *et al.*, 1988). In trypanosome infected cattle and rabbits, cell-mediated DTH responses against trypanosomal antigens have been detected which is suggestive of T cell response (Tizard and Soltys, 1971; Mansfield and Kreier, 1972; Emery *et al.*, 1980c).

The significance of the presence of $CD8^+$ cells in medullary areas of lymph nodes draining local skin reactions is not clear from this study. However, if the functions of these cells in sheep are similar to those of rat, it is possible that they do play an immunosuppressor role as well as contribute to the marked expression of MHC Class II antigens in lymph nodes. *In vivo* depletion of $CD8^+$ cells in rats infected with *T.b. brucei* results in abrogation of interferon-gamma (IFN-y) production, suppression of parasite growth and enhanced survival (Bakheit *et al.*, 1990). This indicates that these cells are responsible for IFN-y production which is a potent cytokine in induction of expression of MHC Class II antigen on cells (Rosa and Fellows, 1984; Skoskiewicz *et al.*, 1985; Kaye, 1987). IFN-y also inhibits macrophage-mediated antigen-specific T cell proliferation (McKernan *et al.*, 1988). This might contribute to the generalized immunosuppression observed in African trypanosomiasis (Askonas and Bancroft, 1984; Murray *et al.*, 1974b) and facilitate the escape of trypanosomes from the host defence mechanisms.

CHAPTER SIX

MIGRATION OF *T. CONGOLENSE* FROM LOCAL SKIN REACTIONS IS ACCOMPANIED BY ALTERATIONS IN CELLULAR PHENOTYPE DYNAMICS IN AFFERENT LYMPH

6.1 Introduction

The cell traffic through areas of inflammation, DTH reactions and allografted tissues in the skin, has been studied by collecting lymph from cannulated afferent lymphatic ducts of sheep (Hay, 1970; Smith *et al.*, 1970; Hall, Lachmann and Trnka, 1973). However, these ducts are fine and therefore difficult to cannulate and maintain lymph flow over a long period. This problem has been overcome by ablation of appropriate lymph nodes and cannulation of the efferent duct six weeks later (Hopkins *et al.*, 1985; Bujdoso *et al.*, 1989; Hopkins *et al.*, 1989). During this time a pseudoafferent vessel is formed when the true afferent lymphatic vessels reanastomose with the former efferent duct. Lymph collected from these lymphatic vessels has a cellular composition similar to that of true afferent lymph (Morris and Coutrice, 1977; Hopkins *et al.*, 1985; Hein *et al.*, 1987).

Afferent lymph from the skin of normal sheep contains a variety of cells including lymphocytes, macrophages and 'veiled' or dendritic cells (Smith *et al.*, 1970). The proportion of SIg^+ lymphocytes is lower than that of blood or efferent lymph (Scollay, Hall and Orlans, 1976; Miller and Adams, 1977). The majority of lymphocytes are T cells which express CD5 antigen and also express detectable levels of MHC Class II antigens (Mackay *et al.*, 1988; Hopkins *et al.*, 1989). Macrophages and veiled cells form 5 to 20% of cells in afferent lymph (Hopkins *et al.*, 1985; Budjoso *et al.*, 1989). These cells express both MHC Class II and CD1 surface antigens (Bujdoso *et al.*, 1989; Hopkins *et al.*, 1989), while 50% of these cells are $CD4^+$ (Mackay *et al.*, 1988).

The composition of afferent lymph is altered by antigenic stimulation of the drainage area such that it contains granulocytes, red blood cells and antigen-antibody complexes (Hall and Morris, 1965; Hein *et al.*, 1987). The cell output and composition also changes. Lymphocyte output in peripheral afferent lymph draining granulomatous and DTH lesions increases (Smith *et al.*, 1970; Hay, Lachman and Trnka, 1973) while increased neutrophil output is a characteristic feature of acute inflammatory reactions elicited by contact with DNFB (Hall and Smith, 1971). This

alteration in composition of afferent lymph is accompanied by changes in cellular phenotype dynamics (Hopkins *et al.*, 1989).

Rapid multiplication of *T. congolense* within the skin causes marked changes in cellular composition. These events are accompanied by enlargement of the draining lymph node as a result of increase in B cell population. The following experiments were designed to examine if these changes are brought about by passage of cells from the skin into the lymph node or by direct stimulation of lymph nodes by invading trypanosomes and also to examine the cellular phenotype dynamics and parasite kinetics in pseudoafferent lymph draining from local skin reactions.

6.2 Materials and Methods

Four sheep were inoculated with 2 x 10^5 culture-derived metacyclic forms of *T. congolense* TREU 1457 or TREU 1881 in 0.2 ml of PSG into the skin area drained by the cannulated pseudoafferent duct. Two inoculations were made on either side of the surgical wound and the sites marked with a black felt-tipped pen. One of the sheep (680) was treated with Berenil (7 mg/kg) 21 days after infection with TREU 1457 and then challenged seven days later with the homologous *T. congolense* serodeme.

Immunofluorescence and flow cytometric analysis of afferent lymph cells was carried out as described in Section 3.8. Two colour immunofluorescence analysis for expression of MHC Class II antigen on T lymphocyte subpopulations ($CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$), B lymphocytes ($CD45R^+$, SIg^+) and $CD1^+$ cells was performed to determine the proportions of activated cell populations. In order to identify the phenotypes of responding cell populations, blast cells and large cells were analyzed by setting gates for cells with higher FSC and SSC. Cellular phenotype dynamics are presented as changes in cell output per hour. However, the sequential alterations in proportions of each cellular phenotype for each sheep is given in Appendix III.

6.3 Results

Lymph flow from one sheep (680R) ceased eight days after infection. However, in the other three sheep lymph was collected for at least 20 days.

6.3.1 Trypanosome kinetics

Trypanosomes were detected in afferent lymph draining local skin reactions from four to six days after infection with culture-derived metacyclic forms of *T. congolense* TREU 1457 (Figure 6.1). Exceptionally, a few trypanosomes were found (sheep 016) in the lymph on day one and two post-infection. Peak parasitosis in lymph (1.6×10^7 trypanosomes per ml) occurred seven to nine days p.i. During this period approximately 1.97 to 10^9 trypanosomes were present in the lymph collected over a period of 24 hours. Parasitosis in each of the sheep infected with *T. congolense* TREU 1457 persisted in lymph and although numbers declined, they never fell below 1.6×10^4/ml throughout the experimental period. Lysed or 'ghost' trypanosomes were seen in lymph during the second and third week of infection. In sheep 680L infected with *T. congolense* TREU 1881, trypanosomes were not detectable microscopically in lymph beyond day nine after infection and peak numbers were only in the order of 6.3×10^4 per ml.

6.3.2 Effect of infection and development of local skin reactions on cell output in afferent lymph

The cell output of sheep 016, following infection with *T. congolense* TREU 1457 is represented in Figure 6.1 and those of the remaining sheep are given in Appendix III. Cell output from uninfected skin ranged between 7.3 to 16.7×10^6/hr. More than 90% of these cells were small lymphocytes while the rest were either macrophages or dendritic ('veiled') cells. Erythrocytes were present most frequently during the first four days after surgery and thereafter were rarely present. Neutrophils were present only in low numbers and rarely exceeded 1×10^5/hr (1%) throughout the course of infection.

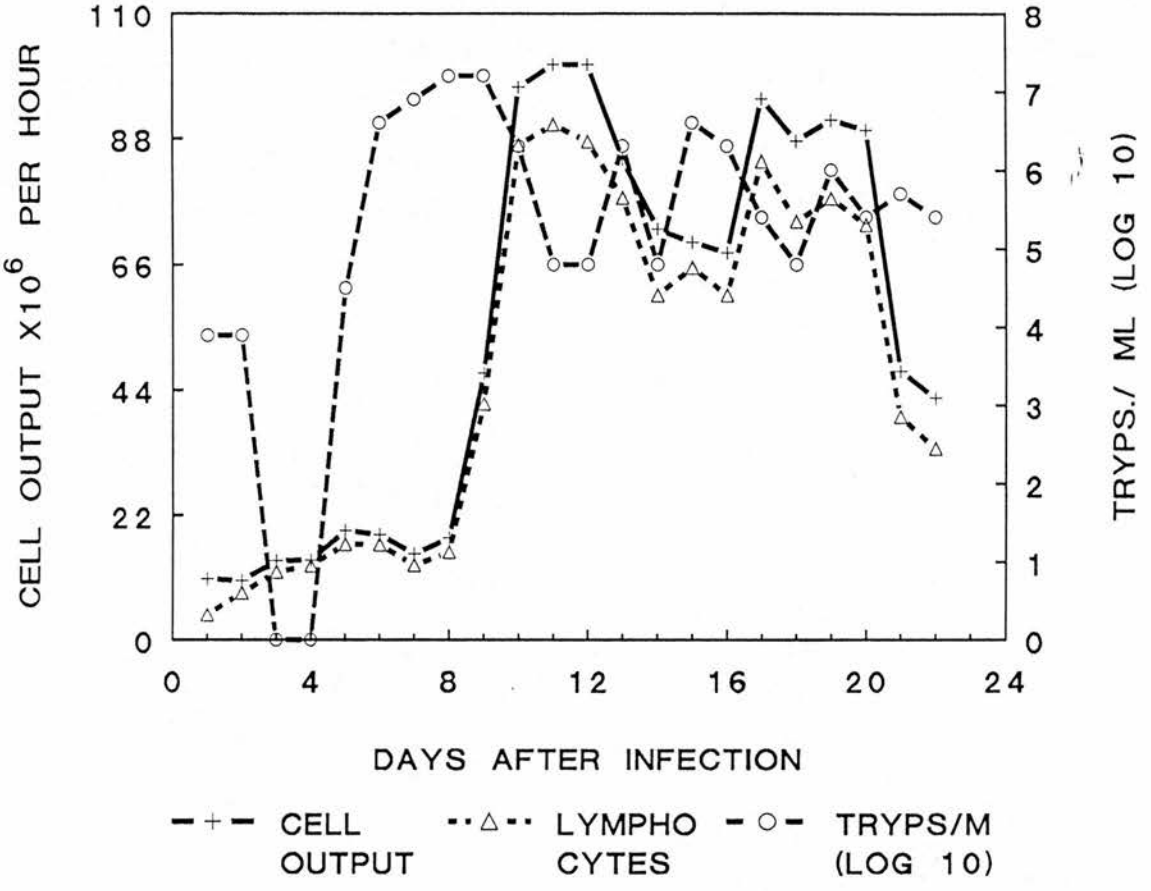
In sheep infected with *T. congolense* TREU 1457 local skin reactions appeared at the inoculation sites from five days p.i. coinciding with the onset of parasitaemia

FIGURE 6.1 Representative example of changes in cellular output of peripheral (afferent) lymph draining from local skin reactions in sheep (016) following intradermal inoculation with metacyclic forms of *T. congolense* TREU 1457.

(A) Graph showing changes in parasite kinetics and the rate of total cell and lymphocyte output.

(B) Graph showing the changes in the rate of output of macrophages, lymphoblasts and granulocytes.

6.1 A


CELL OUTPUT X10^6 PER HOUR
TRYPS./ ML (LOG 10)
110
88
66
44
22
0
8
7
6
5
4
3
2
1
0
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8
12
16
20
24
DAYS AFTER INFECTION
CELL OUTPUT
LYMPHO CYTES
TRYPS/M (LOG 10)

6.1B

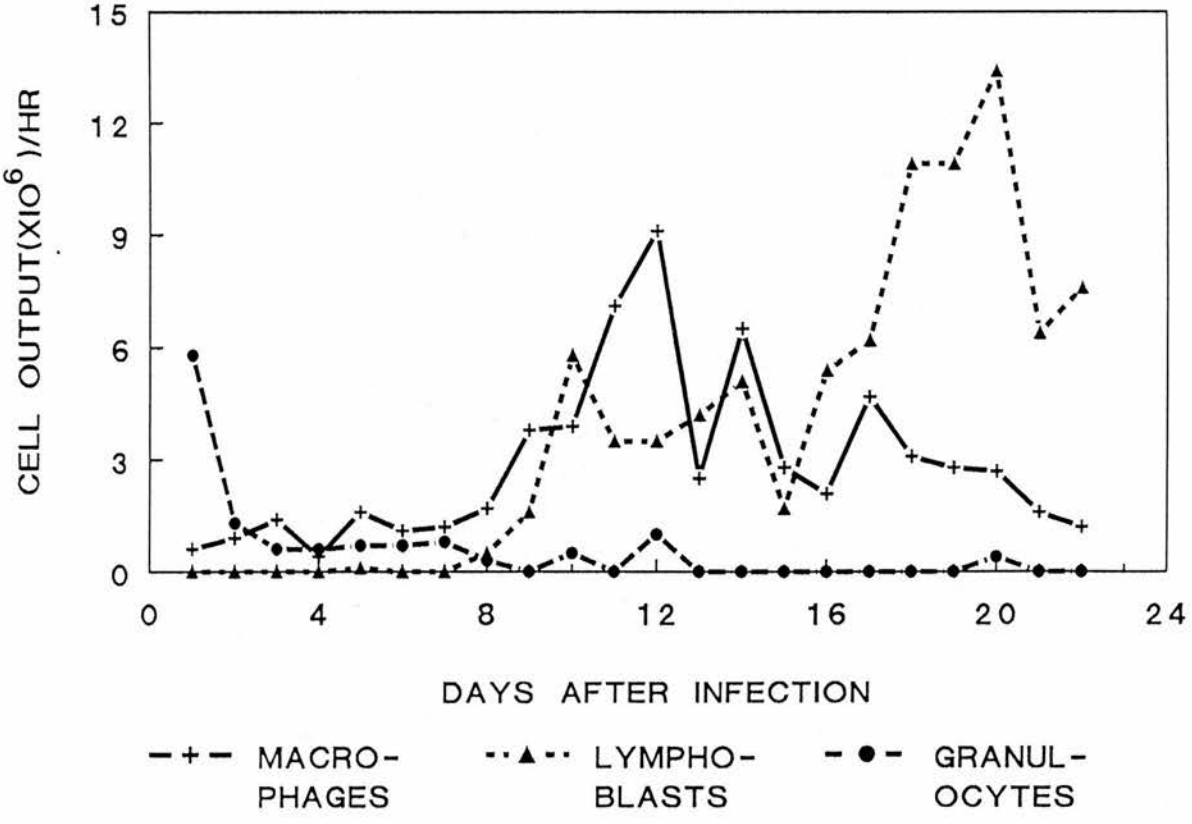
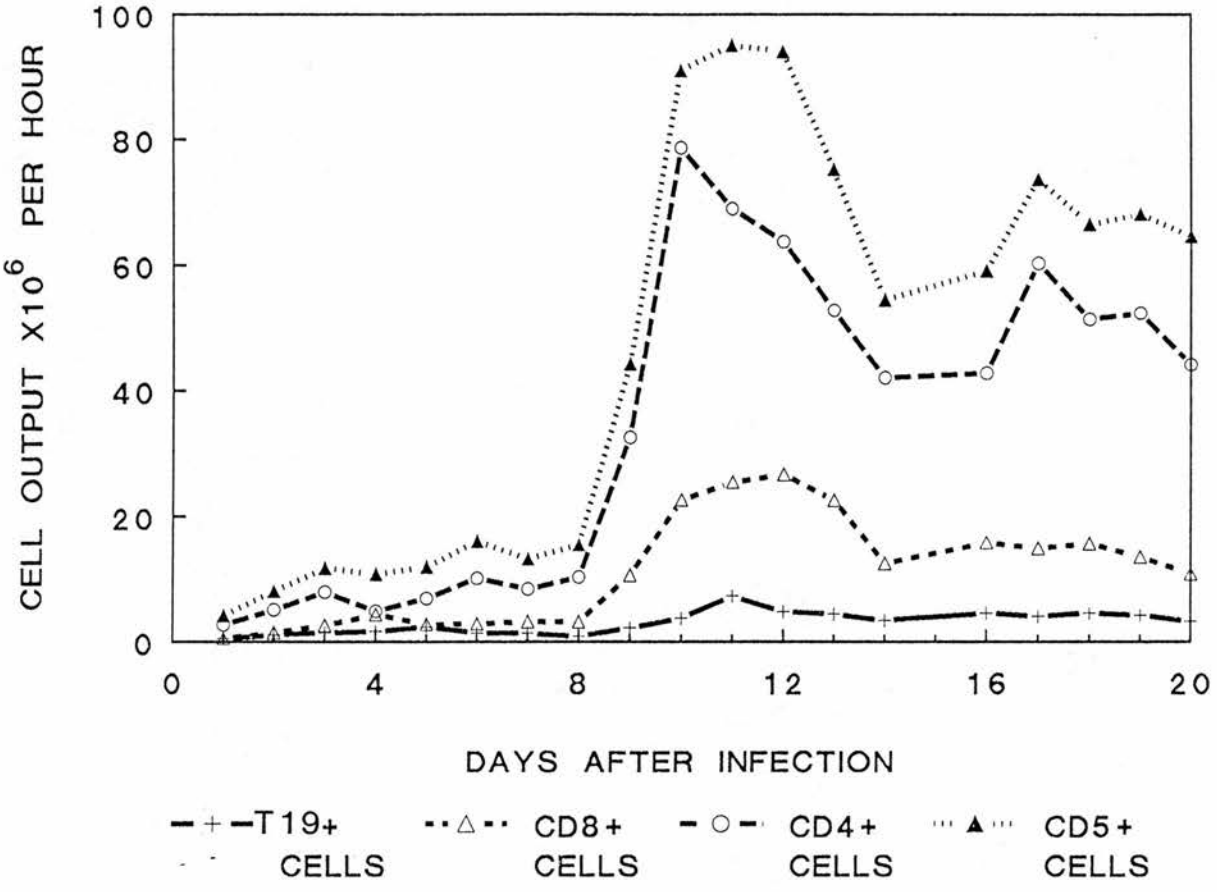

CELL OUTPUT(XIO^6)/HR
15
12
9
6
3
0
0
4
8
12
16
20
24
DAYS AFTER INFECTION
MACRO-PHAGES
LYMPHO-BLASTS
GRANUL-OCYTES

FIGURE 6.1 Representative example of changes in cellular output of peripheral (afferent) lymph draining from local skin reactions in sheep 016 following intradermal inoculation with metacyclic forms of *T. congolense* TREU 1457,

(C) Graph showing dynamics of T cell subpopulations ($CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$ cells) in afferent lymph draining from local skin reactions.

(D) Graph showing the dynamics of surface immunoglobulin bearing cells (SIg^+) MHC Class II^+, $CD1^+$ and $CD45R^+$ cells in the afferent lymph draining from local skin reaction.

6.1C


100
80
60
40
20
0
CELL OUTPUT X10^6 PER HOUR
0
4
8
12
16
20
DAYS AFTER INFECTION
T19+ CELLS
CD8+ CELLS
CD4+ CELLS
CD5+ CELLS

6.1D

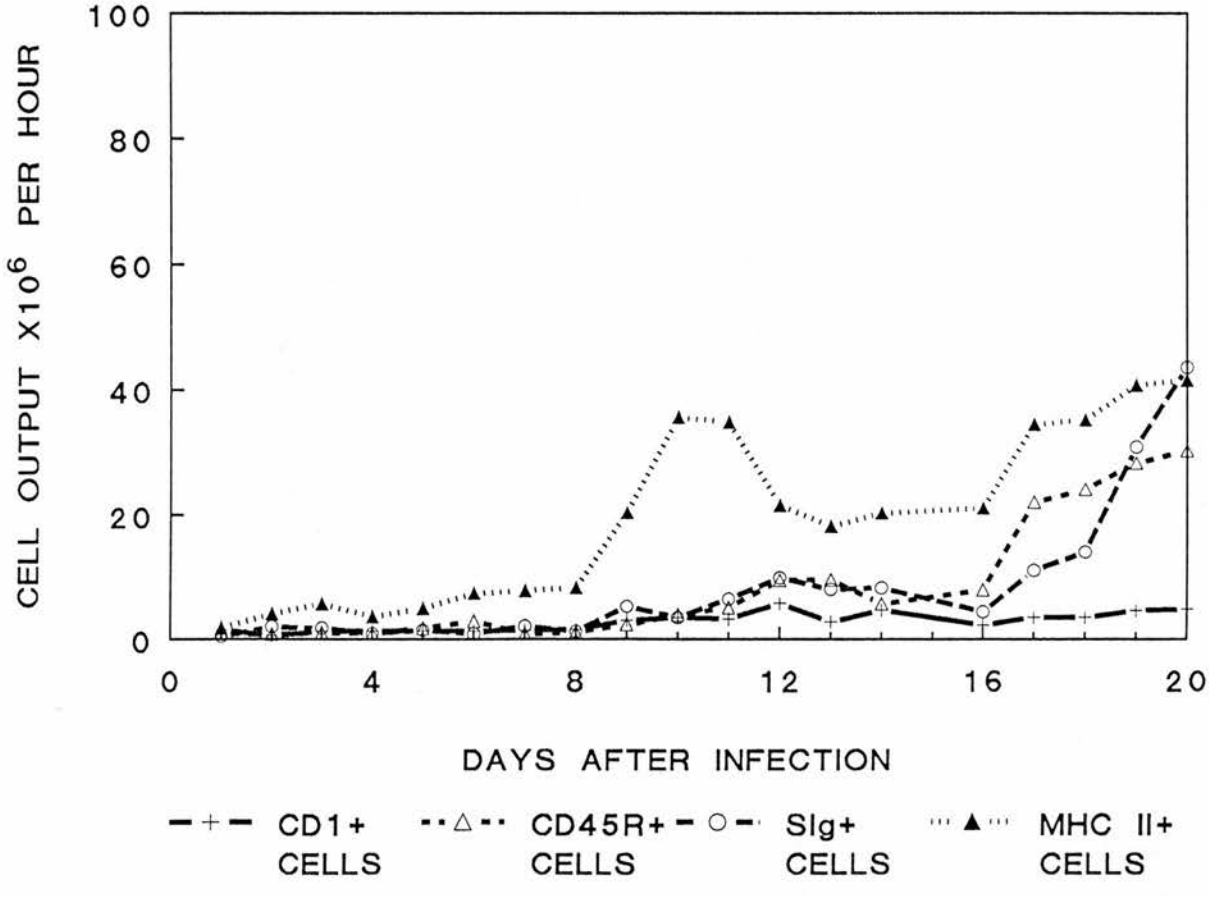
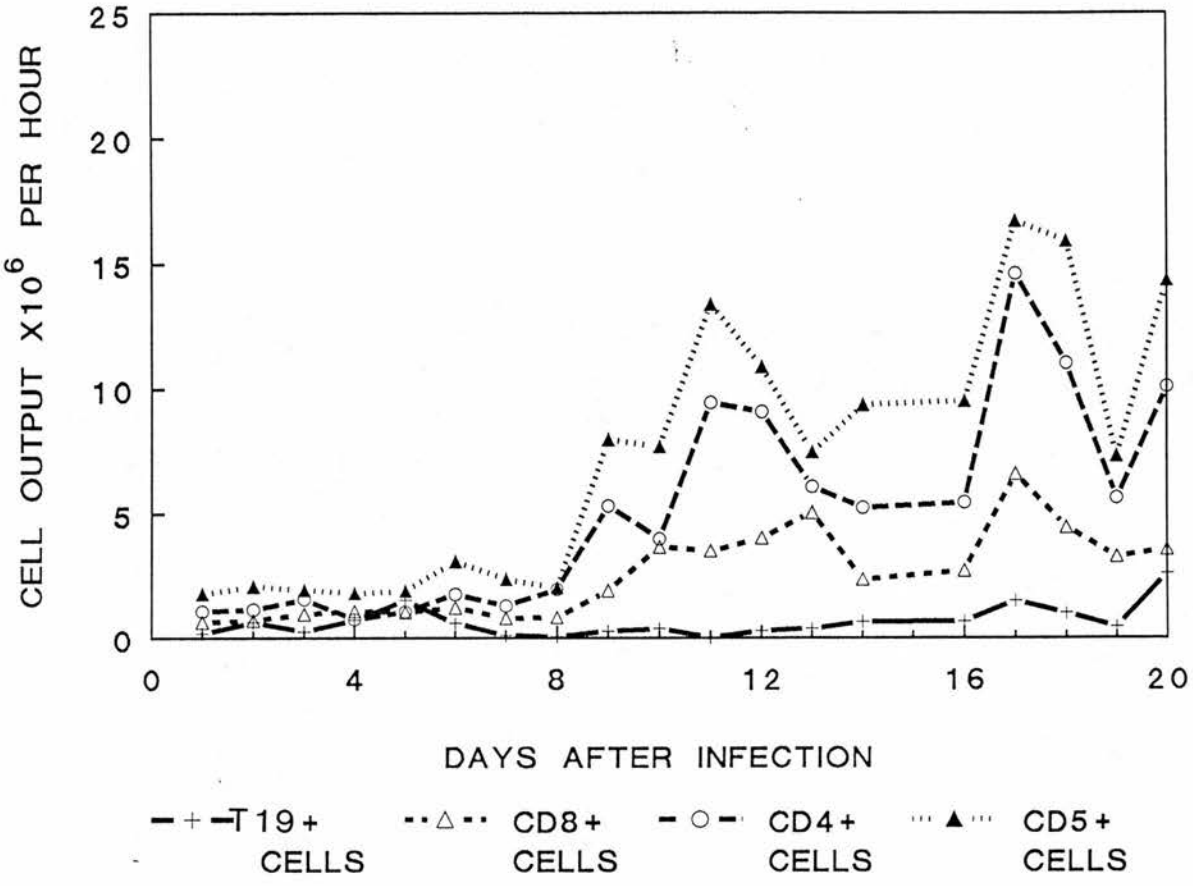

100
80
60
40
20
0
CELL OUTPUT X10^6 PER HOUR
0
4
8
12
16
20
DAYS AFTER INFECTION
CD1+ CELLS
CD45R+ CELLS
SIg+ CELLS
MHC II+ CELLS

FIGURE 6.1 Representative example of changes in cellular output of afferent lymph draining from local skin reactions in sheep 016 following intradermal inoculation with metacyclic forms of *T. congolense* TREU 1457.

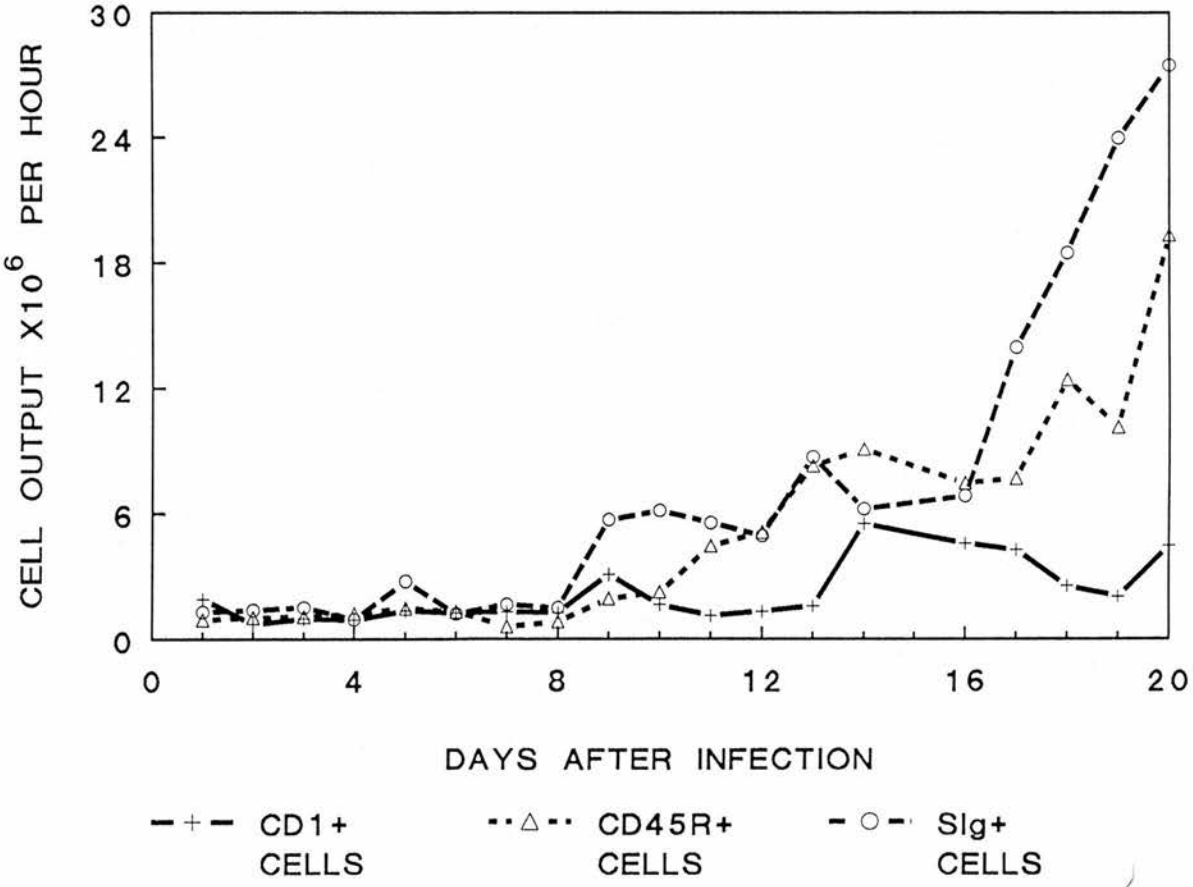
(E) Graph showing the dynamics of T cell subpopulations ($CD5^+$, $CD4^+$, $CD8^+$, and $SBU\text{-}T19^+$ cells) expressing MHC Class II antigens in afferent lymph draining from local skin reactions.

(F) Graph showing the dynamics of $CD1^+$, $CD45R^+$ and SIg^+ cells expressing MHC Class II antigens in afferent lymph draining from local skin reactions.

6.1E


CELL OUTPUT X10^6 PER HOUR
25
20
15
10
5
0
0
4
8
12
16
20
DAYS AFTER INFECTION
T19+ CELLS
CD8+ CELLS
CD4+ CELLS
CD5+ CELLS

6.1F


CELL OUTPUT X10^6 PER HOUR
30
24
18
12
6
0
0
4
8
12
16
20
DAYS AFTER INFECTION
CD1+ CELLS
CD45R+ CELLS
SIg+ CELLS

and increase in cell output in afferent lymph (Figure 6.1). The cellular response was biphasic, with the initial increase occurring during the early part of the second week after infection (seven to 12 days). The second phase occurred during the third week (14 to 17 days) coinciding with the regression of the local skin reaction. In both cases, the increase in cellular output was five to nine times that of pre-infection values. The cellular response, especially in the latter phase, was characterized by an increase in both the percentage and absolute numbers of lymphoblasts (Figure 6.1) from a resting level of 0.5% to 17.8%. There was no overall change in the proportions of macrophages/veiled cells in the lymph although an increase in the numbers of these cells was observed coincident with the increase in total cell output (Figure 6.2). The cellular output in afferent lymph of sheep (680L) infected with *T. congolense* TREU 1881 increased from day four with the peak on day six after infection (day of onset of parasitaemia). A decline to pre-infection levels was observed by days 10 and 11, followed by a second phase of increased cellular output from 12 to 20 days after infection.

6.3.3 Effect of infection and development of local skin reactions on T lymphocyte subpopulations in afferent lymph

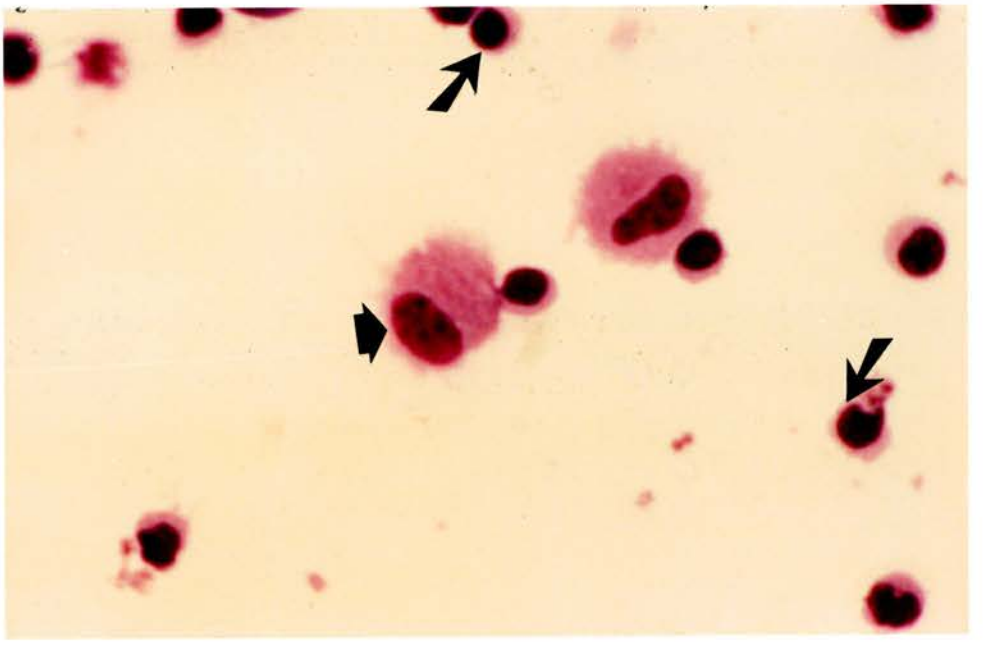
The most notable alteration in cell dynamics in sheep infected with TREU 1457 involved T lymphocyte subpopulations (Figure 6.1 and 6.3). Prior to infection, the majority of lymphocytes (60 to 80%) expressed pan-T cell antigen (CD5). $CD4^+$ cells comprised 35.8 to 52.7%, $CD8^+$ cells 10.8 to 17.2% and SBU-$T19^+$ cells 8.2 to 20.3% of the total cell population. Following infection, the proportions of $CD5^+$ and $CD4^+$ cells markedly increased after onset of parasitosis but later declined as the local skin reaction regressed. A similar trend, but to a lesser degree was observed for $CD8^+$ cells while the proportions of $T19^+$ cells decreased markedly over time to as low as 3.9% in some cases. Representative flow cytometry profiles are shown in Figure 6.3. Coupled with the changes in total cell and lymphocyte output, the T cell response was that of biphasic increase in output of $CD5^+$, $CD4^+$ and to a lesser extent $CD8^+$ cells with very minimal increase in absolute numbers of SBU-$T19^+$ cells.

FIGURE 6.2

(A) Cells in afferent lymph draining from uninfected skin of sheep. The lymph contains mainly small lymphocytes (arrows) and macrophages/dendritic cells (arrow head). Giemsa x500.

(B) Cells and trypanosomes in the afferent lymph draining from a local skin reaction 10 days after infection with *T. congolense* TREU 1457. Numerous trypanosomes and lymphoblasts are present. Giemsa x500.

A



B

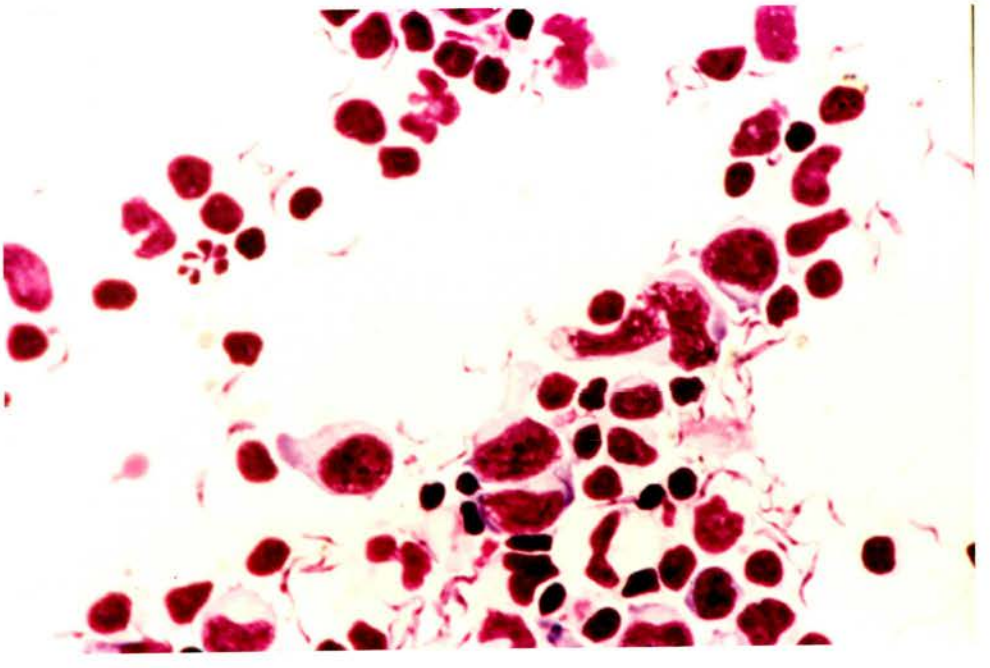


FIGURE 6.3 Representative flow cytometry profiles of T cell subpopulations in afferent lymph draining from local skin reactions in sheep, three, 10 and 20 days after infection with *T. congolense* TREU 1457. The proportion of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

(A) $CD5^+$ cells showing a marked increase 10 days after infection.

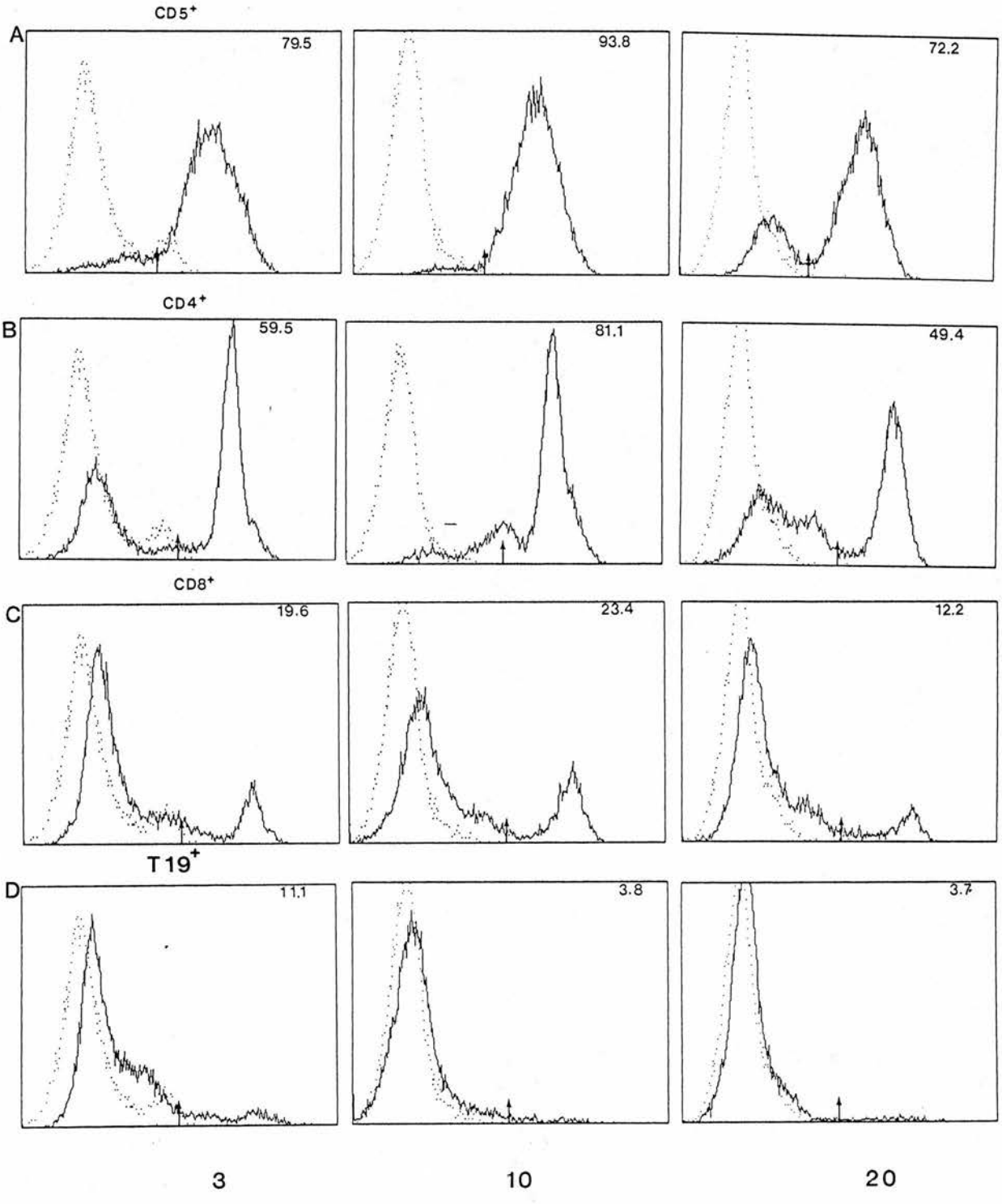
(B) $CD4^+$ cells showing a marked increase 10 days after infection.

(C) $CD8^+$ cells. A slight increase was observed 10 days after infection but then declined on day 20 after infection.

(D) SBU-$T19^+$ cells. A marked decrease was observed during the course of infection.

The dotted profile in each case represents a negative control cell sample while arrows indicate the distinction between negative and positive cells.

6.3



Days after infection

Changes in proportions and absolute numbers of T lymphocyte subpopulations in the sheep infected with *T. congolense* TREU 1881 were not as marked as those of sheep infected with *T. congolense* TREU 1457. The proportions of $CD5^+$ cells declined from 76.6% on day five to 53.7% on day 16 after infection and then increased to above 67% from 18 to 21 days after infection. The absolute numbers also declined seven to nine days after infection. Proportions of $CD4^+$ cells increased from 34.3% (day four) to above 41% between five to six days after infection and coincided with a two- to three-fold increase in cell output. Proportions of $CD8^+$ cells increased only marginally during this period but showed a two-fold increase in absolute cell output. This was followed by a decline in both the proportion and absolute $CD8^+$ cell outputs between nine to 15 days p.i. Both the proportions and absolute output of SBU-$T19^+$ cells declined from six days after infection.

6.3.4 Effect of infection and development of local skin reactions on the dynamics of SIg^+ and $CD45R^+$ cells in afferent lymph

Prior to infection, afferent lymph contained between 4.9 to 23.0% SIg^+ cells, and 8.2 to 18.9% $CD45R^+$ cells. The majority of these cells were presumably B cells. Following infection with *T. congolense* TREU 1457, little change was observed in their proportions except for a slight increase during the third week of infection which coincided with regression of the local skin reaction (Figure 6.4). However, due to the increase in cell output during this period , the absolute numbers of $CD45R^+$ and SIg^+ cells increased (Figure 6.1). This increase in B cell output coincided with the increase in number of blast lymphocytes. In sheep infected with *T. congolense* TREU 1881 no change in proportions of SIg^+ and $CD45R^+$ cells occured until 12 days p.i. when a marked increase was observed. A transient increase in absolute numbers of cells was observed five to eight days p.i. followed by a second increase from 12 days p.i.

6.3.5 Effect of infection and development of local skin reactions on dynamics of MHC Class I^+, MHC II^+, $CD1^+$ and $CD45^+$ cells in afferent lymph

More than 98% of cells in the lymph expressed both MHC Class I and leucocyte common antigens, LCA (CD45). Prior to infection 28.4 to 49.8% and 19.2

FIGURE 6.4 Representative flow cytometry profiles of cells in afferent lymph draining from local skin reactions in sheep three, 10 and 20 days after infection with *T. congolense* TREU 1457. The proportion of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

(A) $CD1^+$ cells declined over time after infection.

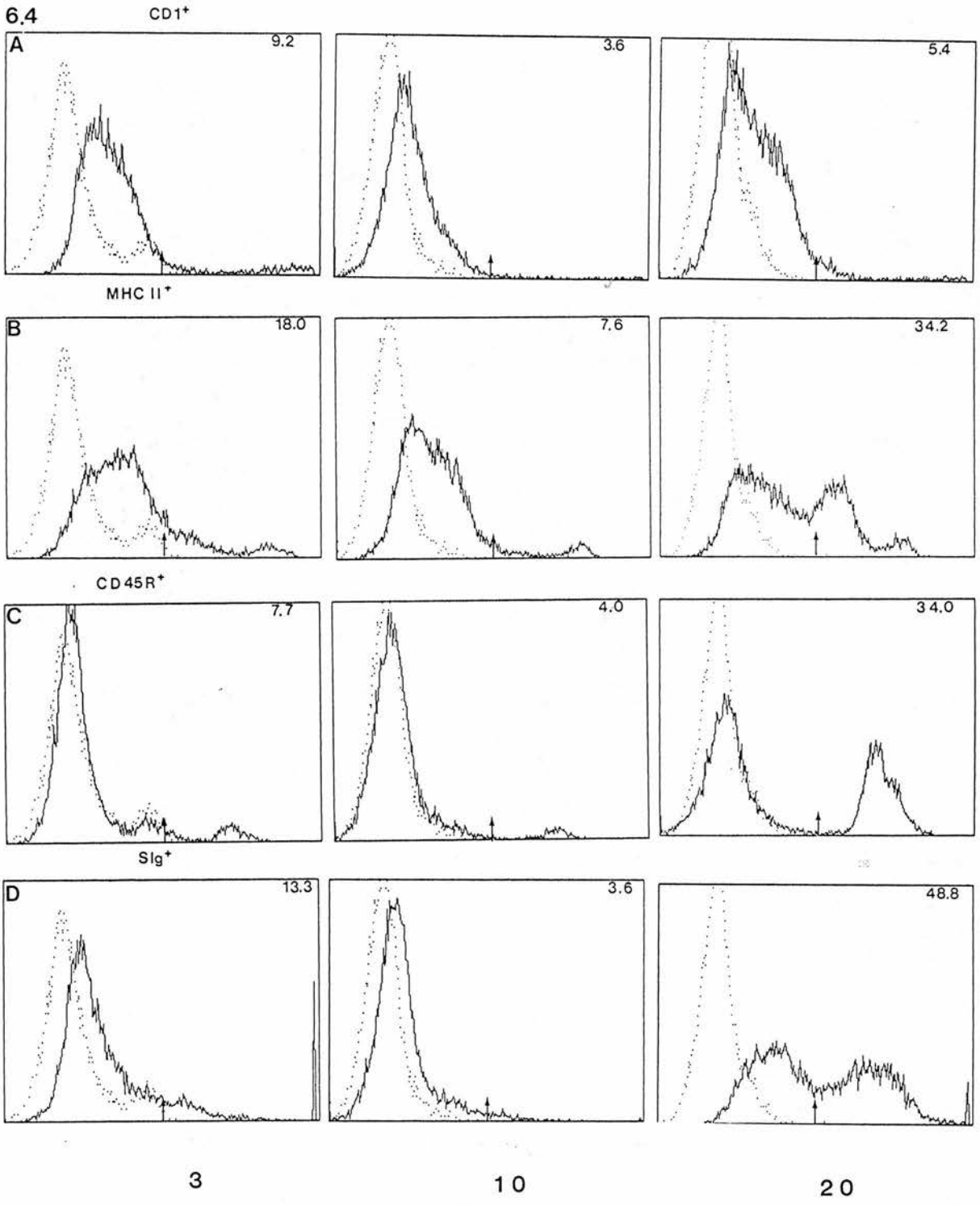
(B) MHC Class II^+ cells declined by day 10 but showed a marked increase 20 days after infection.

(C) $CD45R^+$ cells declined by day 10 but showed a marked increase 20 days after infection.

(D) SIg^+ cells declined on day 10, but showed a marked increase 20 days after infection.

The dotted profile in each case represents the negative control cell sample while the arrows indicate the distinction between negative and positive cells.

6.4



Days after infection

to 41.7% expressed the two markers for MHC Class II antigens, SBU-II and SW73.2 respectively. No major alterations occurred in the proportions of MHC I^+, MHC II^+ and $CD45^+$ cells during infection (Figure 6.4). However, in relation to the absolute numbers, there was an overall increase due to increased cell output. The increase in the absolute number of MHC $Class^+$ II cells was more marked during the second peak of cell output (14 to 17 days) (Figure 6.1).

$CD1^+$ cells expressed high levels of surface MHC Class II antigens and comprised 5.8 to 11.6% of all the cells in lymph from uninfected skin. Following infection, a decrease in their proportions (as low as 3.2%, sheep 016) was observed, but due to an increase in total cell output, the overall trend was of a marginal increase in absolute numbers (Figure 6.1). In the sheep infected with *T. congolense* TREU 1881 no marked changes in proporties of $CD1^+$ and MHC Class II^+ cells was observed. However, increases occurred in the output of $CD1^+$ cells five to eight days p.i. and two phases of increase in absolute numbers of MHC Class II^+ on four to eight days and 12 to 19 days after infection.

6.3.6 Phenotypic characteristics of cells expressing MHC Class II antigens

During the course of infection, there was no distinct trend in alterations of proportions of $CD5^+$ or $CD4^+$ cells which expressed MHC II. There were occasional decreases after onset of the parasitosis. This did not appear to be related to the overall change in cell output. However, a decline in proportions of $CD8^+$ cells and SBU-$T19^+$ cells which expressed MHC II was observed. Similar decreases were observed for $CD1^+$ and $CD45R^+$ cells during the first peak of cell output, however $CD45R^+$, MHC Class II^+ and SIg^+ MHC Class II^+ cells increased markedly during the second phase of cell output (Figure 6.1).

With the exception of SBU-$T19^+$ cells there was a marked increase in absolute numbers of T cells ($CD5^+$, $CD4^+$ and $CD8^+$ cells) which expressed MHC Class II (Figure 6.5). The numbers of sIg^+ and $CD45R^+$ cells expressing MHC Class II

FIGURE 6.5 Two colour immunofluorescence analysis of cells in afferent lymph of sheep infected with *T. congolense* TREU 1457. Representative contour plots of cells collected three, 10 and 20 days after infection stained for expression of various lymphocyte subpopulation markers and MHC Class II. T cell subpopulations were labelled red with phycoerythrin conjugated rabbit anti-mouse IgG. MHC Class II cells were labelled green with FITC conjugated SW73.2 (anti-MHC Class II MAb). The double staining cells are in the upper right quadrant.

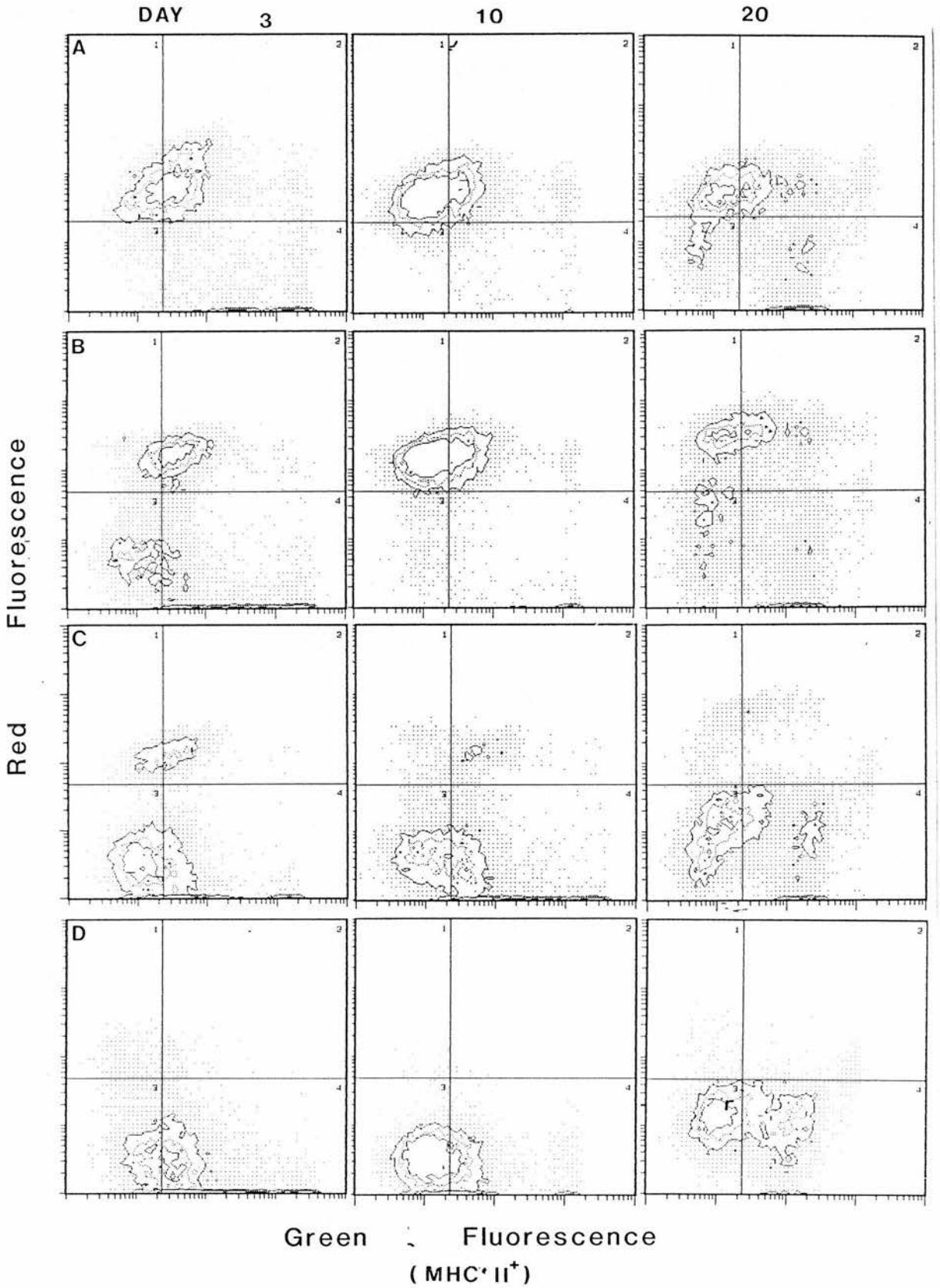
(A) $CD5^+$ cells.

(B) $CD4^+$ cells.

(C) $CD8^+$ cells.

(D) SBU-$T19^+$ T cells.

6.5



increased later on during the regression of chancre. $CD1^+$/MHC II^+ cells showed only a marginal increase in output at the same period (Figure 6.6).

6.3.7 Cellular phenotype dynamics and parasite kinetics in infected, treated sheep challenged with an homologous *T. congolense* serodeme

In sheep 680R which had been treated with trypanocidal drug, no trypanosomes were detected in the lymph after the sheep had been superinfected with the homologous *T. congolense* serodeme seven days after drug therapy. In addition, no increase in cellular output occurred. Little change was observed in both the proportions and absolute numbers of T lymphocytes ($CD5^+$, $CD4^+$, $CD8^+$, SBU-$T19^+$), B cells ($CD45R^+$, SIg^+), MHC Class II^+ and $CD1^+$ cells. A slight decline in the proportion and absolute numbers of $CD5^+$ cells was observed five to seven days after superinfection. The general trend was a gradual decline in the absolute numbers of all cellular phenotypes (Table 3.17 to 3.20 Appendix III).

6.4 Discussion

Cells of afferent lymph are of great interest in the study of the initiation of immune response since they are the first to come into contact with foreign antigens entering through the skin (Issekutz, Chin and Hay, 1980). These cells have migrated from blood into tissues and are travelling towards the regional draining lymph nodes (Hein *et al.*, 1987). This cell traffic through non-lymphoid tissues such as the skin is modest in comparison with that in lymphoid organs, but following antigen stimulation or inflammation the cell traffic is of a similar magnitude to that in lymphoid organs. Afferent lymphatic duct cannulation enables studies to be carried out on the cell traffic through areas of antigen deposition and the movement of parasites from the skin into the circulatory system. The present experimental studies have confirmed suggestions that the major route of migration of trypanosomes from the skin to the systemic circulation is through the afferent lymphatics and draining regional node (Luckins and Gray, 1979; Gray and Luckins, 1980; Akol and Murray, 1982; Akol and Murray, 1986; Dwinger *et al.*, 1990). Large numbers of actively dividing trypanosomes left the skin from seven to 10 days p.i. coincident with maximal development of the

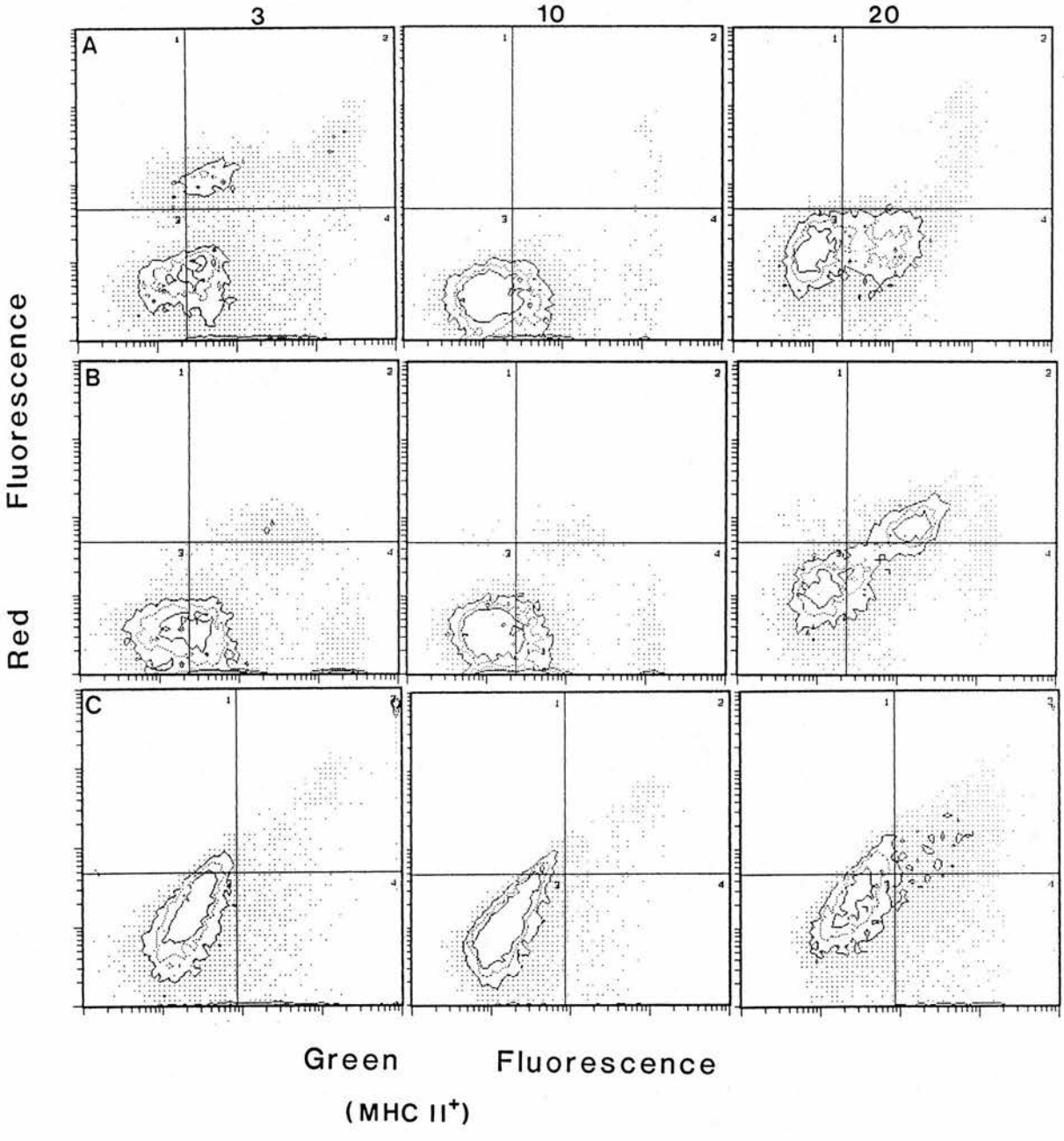
FIGURE 6.6 Two colour immunofluorescence analysis of cells in afferent lymph of sheep infected with *T. congolense* TREU 1457. Representative contour plots of cells collected three, 10 and 20 days after infection stained for expression of various lymphocyte markers and MHC Class II. Double staining cells are in the upper right quadrant.

(A) $CD1^+$/MHC Class II^+ cells. RPE labelled $CD1^+$ cells (red fluorescence) and FITC-SW73.2 labelled MHC Class II^+ cells (green fluorescence).

(B) $CD45R^+$/MHC Class II^+ cells. RPE labelled $CD45R^+$ cells (red fluorescence) and SW73.2 FITC labelled MHC Class II^+ cells (green fluorescence).

(C) SIg^+/MHC Class II^+ cells. RPE labelled MHC Class II^+ cells (red fluorescence) and DAS-FITC Ig labelled SIg^+ cells (green fluorescence).

6.6


Days after infection
3
10
20
A
B
C
Red Fluorescence
Green Fluorescence
(MHC II+)

chancre. This is an indication that active trypanosome proliferation is taking place not only within the skin reaction but also in the afferent lymph.

The lymph flow, cell output and cell composition of lymph from the skin of uninfected sheep was similar to that described by other workers (Hall, 1967; Smith *et al.*, 1970; Hein *et al.*, 1987). Over 90% of these cells were small lymphocytes with the rest being macrophages/dendritic cells. The phenotypes of these cells showed a high proportion of $CD5^+$ cells, low proportion of SIg^+ cells and dendritic cells which was also in agreement with similar studies (Mackay *et al.*, 1986; Hopkins *et al.*, 1989).

Peripheral lymph draining allografted tissue, granulomatous lesions or areas of antigen stimulation in sheep show marked alterations in both lymph flow and cell output (Smith *et al.*, 1970; Hall and Smith, 1971; Issekutz *et al.*, 1980, 1981; Hopkins *et al.*, 1989). The composition of the cells varies with the nature of inflammatory reaction as well as the type of antigenic stimulus. Lymph from acute inflammatory reactions elicited in skin by DNFB is characterized by an increase primarily of neutrophils (Hall and Smith, 1971). However, lymph draining DTH reactions contains predominantly mononuclear cells which is a reflection of cells infiltrating this lesion (Smith *et al.*, 1970; Issekutz *et al.*, 1980; 1981).

The cell output in afferent lymph draining trypanosomal chancres showed some differences from these studies. Increase in cell output in peripheral lymph was observed only on detection of the local skin reaction and coincided with presence of trypanosomes in the lymph. Although trypanosomal chancres histologically contain a large proportion of polymorphonuclear cells (PMNS) early in infection (Akol and Murray, 1982; Dwinger *et al.*, 1987; Chapter Four) no increase in these cells was observed in the lymph. It is probable that neutrophils which extravasate and enter the skin reaction fulfil their function and are destroyed within the lesion. Alternatively, they may have been selectively prevented from entering the lymphatic system. The cell response in afferent lymph was bimodal. The initial phase of increased cell

output coincided with the development of the local skin reaction and cell composition resembled that described following secondary antigen challenge or lymph draining areas of chronic inflammation (Issekutz *et al.*, 1982; Hopkins *et al.*, 1989). This response was essentially due to a marked increase in the proportion and absolute numbers of $CD5^+$ cells especially the $CD4^+$ phenotype. The proportions of SBU-$T19^+$ and $CD8^+$ cells, $CD1^+$, MHC II^+, SIg^+ and $CD45R^+$ cells decreased during this period. However, considering the increase in total cell output, the numbers of $CD8^+$, MHC Class II^+ and macrophage-like cells increased during this period with very marginal increase in SIg^+ and $CD45R^+$ cells. Coincident with this period slight increases in lymphoblasts was observed. Although there were no major changes in $CD5^+$ and $CD4^+$ expressing MHC Class II^+ antigens, they accounted for most of the MHC Class II cells. This indicates that the initial phase of cellular reaction was predominantly due to a T cell response. This agrees with findings that secondary immune responses to purified protein derivative (PPD) and other antigens result in the proportions of SIg^+/MHC Class II^+ cells in lymph remaining unaltered but T cells expressing MHC Class II antigens increasing (Hopkins *et al.*, 1986; Hopkins *et al.*, 1989).

The second phase of the cellular response which occurred during the regression of the skin reaction and was characterized by a marked increase in the proportion of lymphoblasts. The absolute numbers of both sIg^+ and $CD45R^+$ (B cell markers) was higher and comprised more than 50% MHC Class II^+ cells. Although the proportions of $CD5^+$ and $CD4^+$ cells declined, their numbers remained well above pre-infection levels, and during this phase, the highest numbers of T cells expressing MHC Class II were present. Only a marginal increase in $CD1^+$/MHC Class II^+ cells (dendritic cells) was observed at this time.

The disparity in cellular phenotype dynamics during these two phases is intriguing. Different lymphocyte subpopulations show different migratory properties in different tissues. The enrichment of normal peripheral lymph with T cells may be

due to a positive selection of these cells or a negative selection against B cells (Mackay *et al.*, 1988). This mechanism appears to be operative or even enhanced during the initial phase of cellular response. B cells ($CD45R^+$) were present in large numbers in the skin reactions during this early phase of response (Section 4.3). The logical conclusion would be that after being recruited into the lesion, B cells do not immediately migrate into the lymph. However, during the regression of the local skin reactions, these cells leave the lesion in large numbers which contributes to the second phase of cellular response in afferent lymph. This explains why the skin reaction is characterized by presence of a large number of T cells and B cells during the early stages and mainly T cells during the regression phase.

During the response, the SBU-$T19^+$ cells did not appear to participate in this cellular response and in fact the proportions of these cells in peripheral afferent lymph declined. This accords with the findings that these cells were not recruited into the skin reaction in large numbers (Section 4.3). Similar observations have been made in intestinal and gastric mucosa of sheep infected with *Trichstrongylus colubriformis* and *Haemonchus contortus* (Gorrell *et al.*, 1988a, 1988b).

The absence of trypanosomes and any cellular responses to homologous challenge of the sheep which had been infected and treated with trypanocidal drug indicated that immunity is effected at the level of the skin. It is possible that this immunity is mediated by humoral factors in the skin rather than by infiltration of cells into the inoculation site.

The events occurring in peripheral afferent lymph draining local skin reactions appear to be important in the pathogenesis of the disease and development of protective immunity. The massive migration of trypanosomes from skin through the lymph offers a pathway for invasion of the host. However, this is potentially advantageous for the host since it offers an opportunity for close association of the parasite and immunocompetent cells. It appears that during the early stages of infection, both B and T cells proliferate in response to trypanosomal antigens. More

importantly, the response of $CD4^+$ cells precedes that of B cells indicating a possible helper effect on antibody production. In African trypanosomiasis, immunological responses of infected hosts involves both humoral and cellular effector mechanisms (Greenblatt *et al.*, 1983). Protective immunity is however effected at the level of skin (Akol and Murray, 1985, 1986; Taiwo *et al.*, 1990). Animals immunized by cyclical infection followed by chemotherapy are immune to homologous challenge (Akol and Murray, 1985; Luckins *et al.*, 1983; Taiwo *et al.*, 1990). These hosts develop DTH reactions following intradermal challenge with homologous antigens (Emery *et al.*, 1980) indicating that DTH might be an important component of the protective mechanism. DTH is an accumulation of mononuclear cells initiated by T_H1 subset of $CD4^+$ population following secondary challenge with specific antigen. The peripheral lymph contains dendritic or veiled cells which are known to cluster and stimulate resting T cells (Inaba and Steinman, 1984) in addition to transporting antigen to draining lymph nodes (Bujdoso *et al.*, 1989; Harkis *et al.*, 1990). It is possible therefore that stimulation of T cells in trypanosome infections occurs either in the skin reaction or most likely in the peripheral lymph during this period before amplification of the immune response in the draining node. These experiments suggest that the response of the draining lymph node is initiated by influx of both cells and trypanosomes from the local skin reaction.

CHAPTER SEVEN

INCREASED B CELL OUTPUT IN EFFERENT LYMPH FROM LYMPH NODES DRAINING *T. CONGOLENSE* INDUCED LOCAL SKIN REACTIONS IN SHEEP

7.1 Introduction

The immunological events occurring in lymph nodes draining inflammatory lesions and sites of antigen deposition are reflected by the cell content of efferent lymph (Trnka and Cahill, 1980; Hopkins *et al.*, 1986; Bujdoso *et al.*, 1990). In domestic ruminants, the kinetics of lymph node responses to various antigenic stimuli have been investigated by cannulation of efferent lymphatic ducts from such nodes, and determination of the cell output and phenotypes of cells in the lymph (Hall and Morris, 1962; Hall, 1967; Emery and McCullagh, 1980; Kerlin and Watson, 1987; McColgan, Buxton and Miller, 1987). The cellular composition of efferent lymph draining unstimulated lymph nodes is totally lymphocytic: T lymphocytes constitute 70 to 80% while B cells represent 20 to 30%. T lymphocyte subpopulations, $CD4^+$, $CD8^+$ and SBU-$T19^+$ T cells make up 35 to 45%, 12 to 18%, eight to 12% respectively, while MHC Class II^+ cells comprise 20 to 60% (Mackay *et al.*, 1988; Kimpton *et al.*, 1989). The composition and cell content of efferent lymph changes following antigenic or inflammatory stimuli in the tissues drained by the regional node (Hall and Morris, 1962; Hall, 1967; Kerlin and Watson, 1987; McColgan, Buxton and Miller, 1987). The lymph from such nodes contains not only an increased number of lymphocytes but also lymphoblasts (Hall and Morris, 1963; Hall and Morris, 1965; Hall, 1967; McColgan *et al.*, 1987) and in some circumstances, such as acute inflammation, erythrocytes and granulocytes (Hall and Morris, 1965; Smith *et al.*, 1970; Kerlin and Watson, 1987). This change in cellular composition is also accompanied by alterations in the phenotypes of various lymphocyte subpopulations (Hopkins *et al.*, 1986; Bujdoso *et al.*, 1990) depending on the antigenic stimuli. Cells leaving the efferent lymph of an antigen primed lymph node are responsible for the systemic dissemination and amplification of the local immune response.

Trypanosome kinetics and cellular dynamics in efferent lymph have been studied by efferent lymphatic duct cannulation of cattle and goats infected with *T. congolense* (Akol and Murray, 1986; Dwinger *et al.*, 1990) and in goats infected with *T. vivax* (Emery *et al.*, 1980b) or *T. brucei* (Emery *et al.*, 1980b; Barry and Emery,

1984). These studies have shown that marked changes occur in the cellular composition of efferent lymph following development of local skin reactions. However, changes in the cellular phenotypes during this period have not been characterized. In the following experiments, the cellular phenotypes leaving the lymph node draining local skin reactions were characterized by surgical cannulation of prefemoral efferent lymphatic ducts, immunofluorescence staining of cells and flow cytometry.

7.2 Materials and Methods

7.2.1 Trypanosomes and infection of sheep

The prefemoral efferent lymphatic ducts of five sheep (742, 749, 758, 767 and 770) were cannulated as described in Section 3.6. The sheep were infected with 2 x 10^5 culture-derived metacyclic forms of *T. congolense* TREU 1457. Two inoculations were made on either side of the surgical site. Three of the sheep (742, 749 and 767) were infected five days after cannulation. Since in these experiments it was found that no alterations in cellular response occurred within the first five days. Sheep 758 and 770 were infected on the same day as lymphatic duct cannulation in order to monitor the response for a longer period. One of the sheep, (758) was superinfected on day 21 with an heterologous *T. congolense* serodeme (TREU 1881) in order to obtain preliminary results on interference of establishment of secondary infections. Data presented in the results section are based on sheep 758 and 749 in which observations on primary infections were made for 14 and 21 days respectively. The detailed data of the other sheep are in the Appendix IV (page 249) with the exception of sheep 767 in which lymph flow ceased soon after infection.

7.2.2 Lymph collection and immunofluorescence staining

Lymph was collected and processed as described in Section 3.6 except that occasionally, due to the large volume of lymph output, the collecting bottle had to be changed after 12 hours. Such lymph, collected at the end of the day was kept at +4°C and then mixed with the portion of lymph collected the following morning.

Immunofluorescence and flow cytometric analysis of efferent lymph cells was carried out as described in Section 3.8. Two colour immunofluorescence analysis for expression of MHC Class II antigens on T lymphocyte subpopulations ($CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$) and B lymphocytes ($CD45R^+$, SIg^+) was performed to

determine activated cell populations. The results are presented as changes in absolute cell output (per hour). However, sequential alterations in proportions of each cellular phenotype for each sheep is given in Appendix IV.

7.3 Results

7.3.1 Trypanosome kinetics

Local skin reactions developed at the two inoculation sites from five day p.i. and the draining prefemoral lymph nodes became enlarged two days later. Trypanosomes were detected in efferent lymph three to six days after infection (Figure 7.1). Peak parasitosis occurred on day nine in sheep 758 (2.9 x 10^6 per ml) and on day 10 in sheep 749 (3.2 x 10^5 per ml). In sheep 758, the lymph collected over 24 hours on day nine contained a total of 3.97 x 10^8 trypanosomes. Trypanosomes were present in lymph even on day 21 p.i. The level of parasitosis was however, low in the order of 3.1 x 10^3 per ml.

7.3.2 Lymph and total cellular output

Prior to infection the lymph output from the prefemoral efferent lymphatic duct ranged from 115 to 280 mls per day (Appendix IV). The cell output varied with individual sheep between 1.90 to 12.8 x 10^7 per hour (Figure 7.1). More than 99% of the total efferent cell populations were lymphocytes although a few erythrocytes and neutrophils were present in lymph collected up to three days after operation but thereafter were rarely seen.

Following infection, there was a slight decrease in daily lymph output which later stabilized. However, marked alterations occurred in cell output which coincided with development of local skin reactions and enlargement of the draining lymph node (Figure 7.2). Initially, cell output decreased between day three to five p.i. and then from six days p.i. increased. The cellular output showed a bimodal response. The first peak of cellular response in sheep 758 (29.1 x 10^7 per hour) and in sheep 749 (21.7 x 10^7 per hour) was observed on day eight while the second lower peak occurred in sheep 758 on day 15 (13.7 x 10^7) and in sheep 749 on day 12 (18.8 x

FIGURE 7.1 Representative example of changes in the cellular output of efferent lymph from prefemoral lymph node draining a local skin reaction in sheep 758 infected by intradermal inoculation of metacyclic forms of *T. congolense* TREU 1457. The graph shows the changes in parasite kinetics and the rate of output of total lymphocytes and lymphoblasts.

7.1

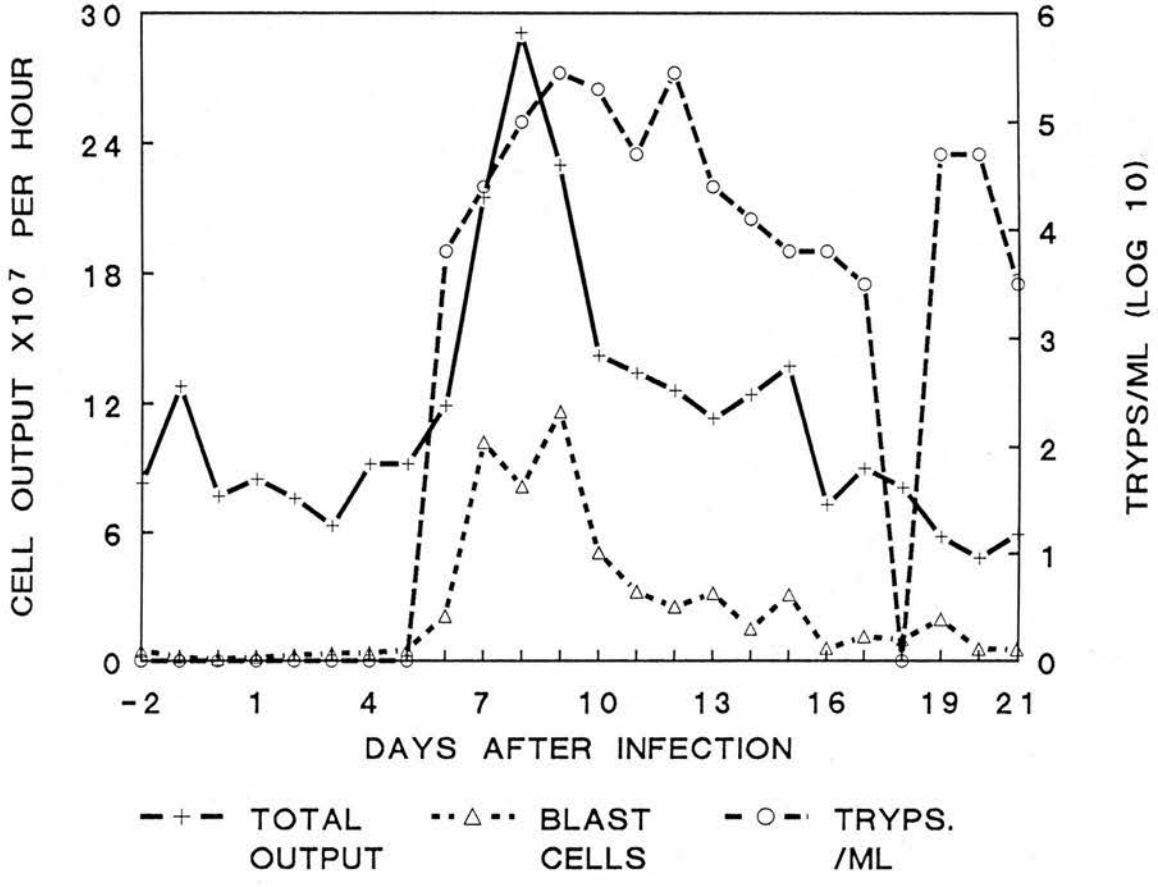
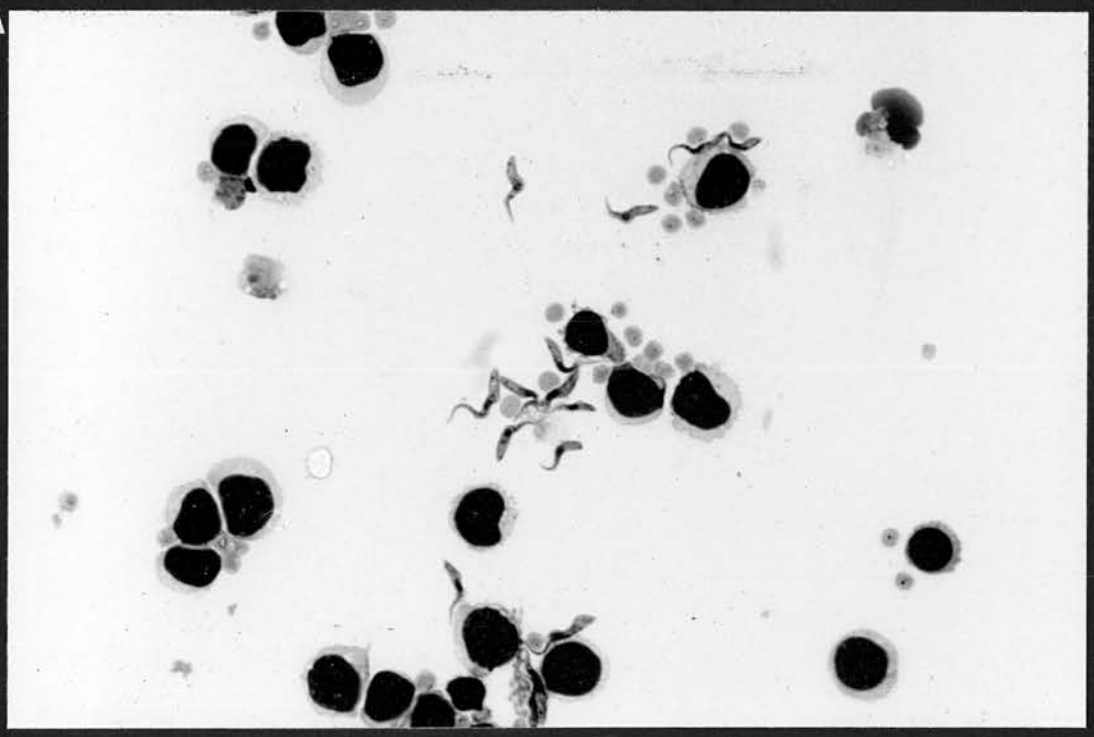

CELL OUTPUT X10⁷ PER HOUR
TRYPS/ML (LOG 10)
30
24
18
12
6
0
6
5
4
3
2
1
0
-2
1
4
7
10
13
16
19
21
DAYS AFTER INFECTION
TOTAL OUTPUT
BLAST CELLS
TRYPS. /ML

FIGURE 7.2 Cytocentrifuge smears of efferent lymphocytes from prefemoral lymph node draining local skin reaction in sheep infected with *T. congolense* TREU 1457.

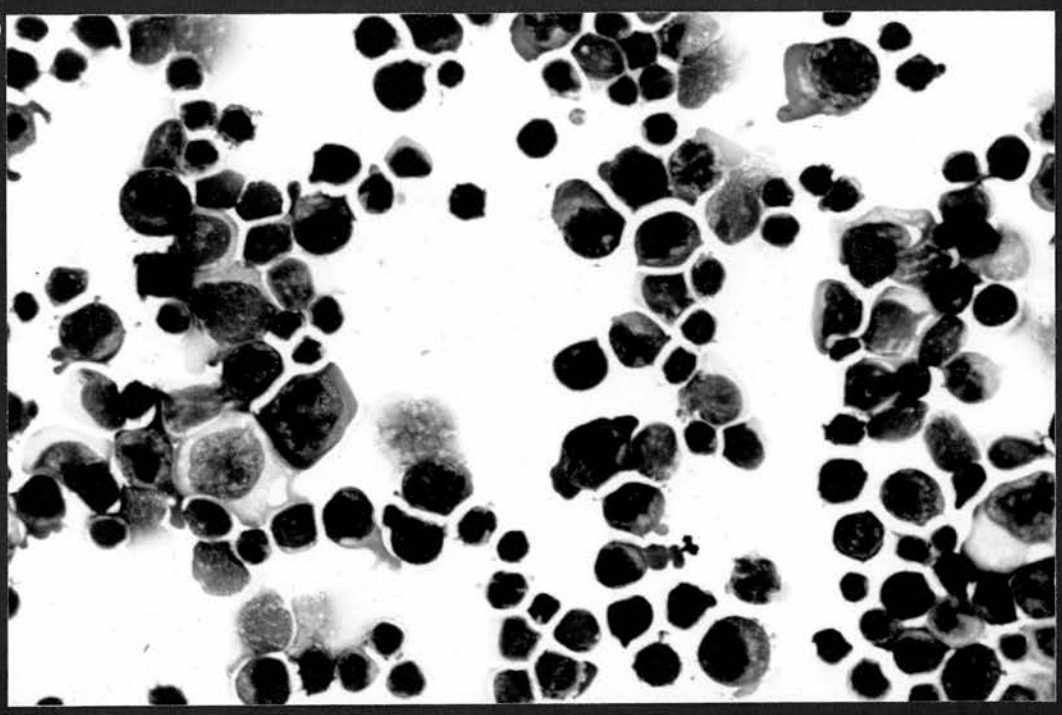
(A) Trypanosomes and small efferent lymphocytes in lymph six days after infection. Giemsa x250.

(B) Numerous lymphoblasts in lymph nine days after infection. Giemsa x250.

A



B



10^7). Thereafter the cellular output declined with the regression of the skin reaction (Figure 7.1 and Appendix IV).

Increase in cellular output was accompanied by an increase in the proportion and absolute numbers of lymphoblasts. Maximum lymphoblast output coincided with the peak of cellular response and was in the order of 4.35 (sheep 749) to 11.62 x 10^7 (sheep 758) per ml of lymph (Figure 7.1).

7.3.3 Alterations in T lymphocyte subpopulations

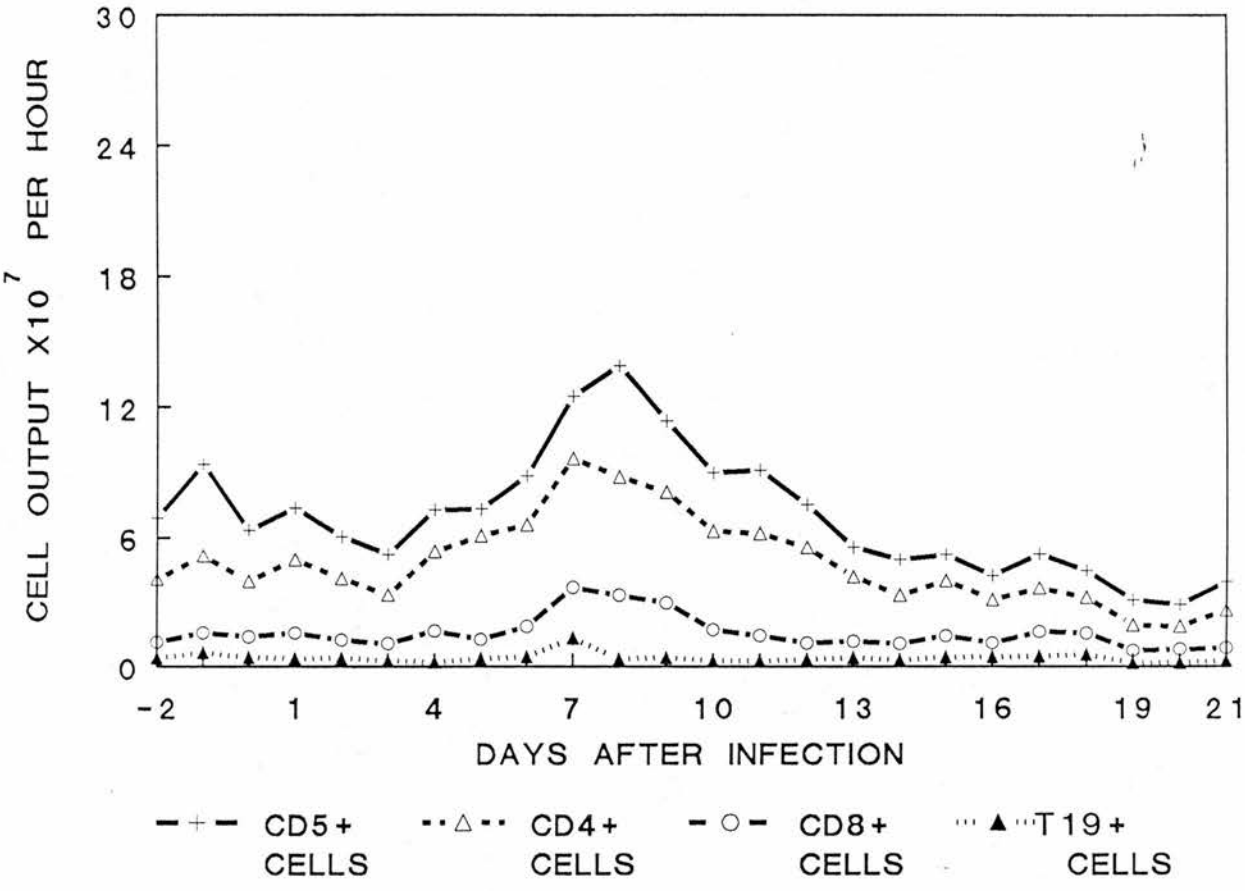
Before infection, the majority of lymphocytes, more than 70%, expressed CD5 antigen at a high level. This population comprised more than 40 to 70% $CD4^+$ cells, 6 to 24% CD8, and 2.5 to 6.7% SBU-$T19^+$ cells. The absolute output of cells ranged between 1.9 to 9.0 x 10^7 per hour for $CD5^+$ cells, 1.13 to 5.0 x 10^7 per hour for $CD4^+$ cells, 0.42 to 1.39 x 10^7 for $CD8^+$ cells and 0.35 to 0.53 x 10^7 for SBU-$T19^+$ cells. Following infection, marked alterations occurred in both the proportion and absolute numbers of T cells in lymph (Figure 7.3 and 7.4). The proportion of $CD5^+$ cells showed a decline from six days p.i. falling as low as 38 to 49.1% between days 13 and 15 p.i. in the two sheep (749 and 758). The proportions of these cells remained low for up to 21 days p.i in sheep 758. A similar decline in the proportions of $CD4^+$ (26.3 to 37.1%), $CD8^+$ (1.8 to 10.4%) and SBU-$T19^+$ (1.2 to 6%) cells was observed on 12 to 15 days p.i. The decrease in absolute T cell output was consistent with decline in their proportion in lymph. The numbers of $CD5^+$ cells decreased initially three to five days p.i. This decline was followed by a two-fold increase in $CD5^+$ cells to 10.4 and 13.9 x 10^7/hour by day eight in sheep 749 and 758 corresponding with an increase in total cellular output (Figure 7.3). Following regression of the skin reaction, a variable but steady decrease was observed. Similar changes to those of $CD5^+$ cells were observed for $CD4^+$ and $CD8^+$ cells, namely an initial decline three to five days after infection followed by a two-fold increase of $CD4^+$ cells to 8.46 to 9.63 x 10^7/hour and $CD8^+$ cells to 3.68 to 1.76 x 10^7/hour on days seven to nine p.i. Numbers of SBU-$T19^+$ T cells remained low. An initial

FIGURE 7.3 Dynamics of cellular phenotypes in efferent lymph from prefemoral lymph node draining local skin reactions in sheep 758 infected with *T. congolense* TREU 1457.

(A) Graph showing the dynamics of T cell subpopulations ($CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$ T cells).

(B) Graph showing the dynamics of SIg^+ MHC Class II^+ and $CD45R^+$ cells.

7.3A


30
24
18
12
6
0
CELL OUTPUT X10 7 PER HOUR
-2
1
4
7
10
13
16
19
21
DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS
T19+ CELLS

7.3B

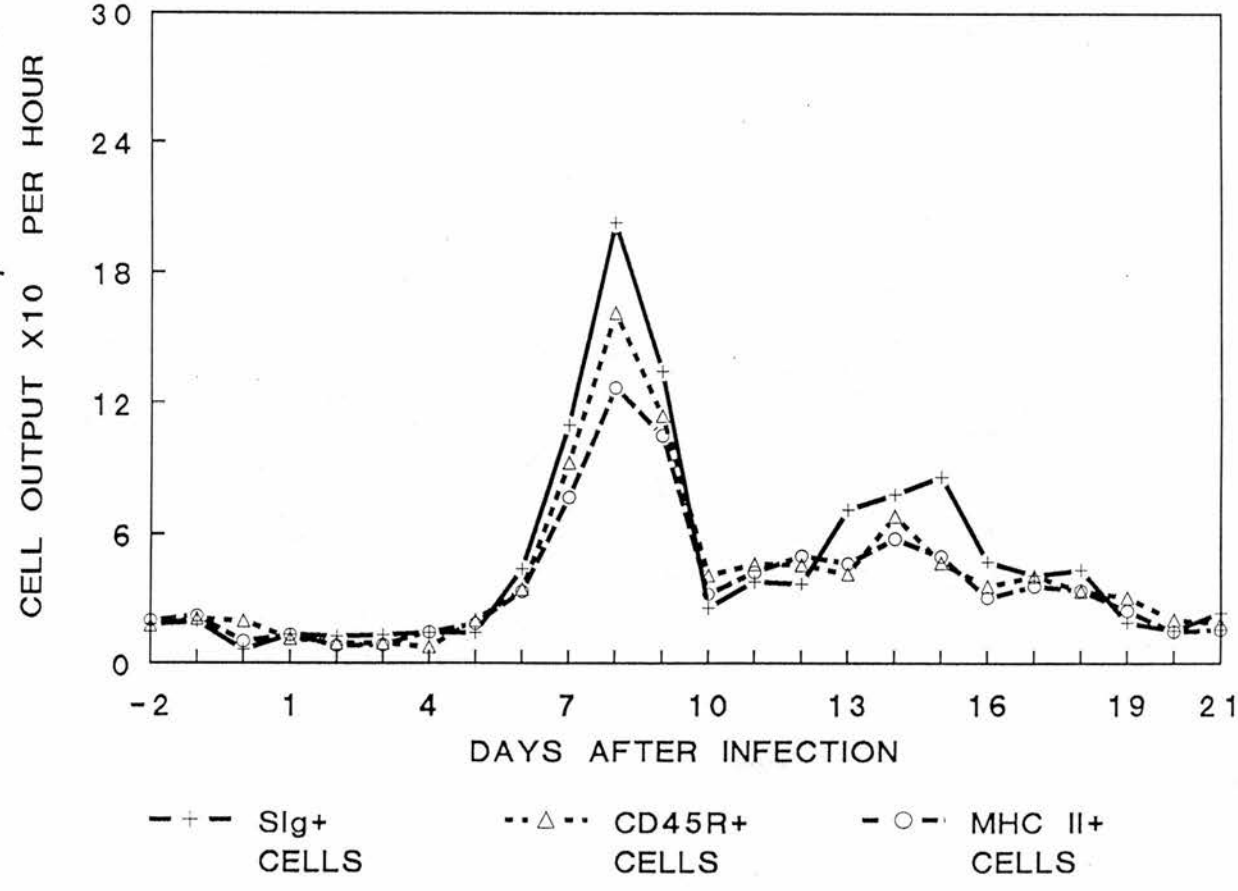

30
24
18
12
6
0
CELL OUTPUT X10 7 PER HOUR
-2
1
4
7
10
13
16
19
21
DAYS AFTER INFECTION
SIg+ CELLS
CD45R+ CELLS
MHC II+ CELLS

FIGURE 7.4 Representative flow cytometry profiles of T cell subpopulations in efferent lymph from prefemoral lymph node draining local skin reactions in sheep 758, two, nine, and 20 days after infection with *T. congolense* TREU 1457. The proportion of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

(A) $CD5^+$ cells showing a marked decrease nine days after infection.

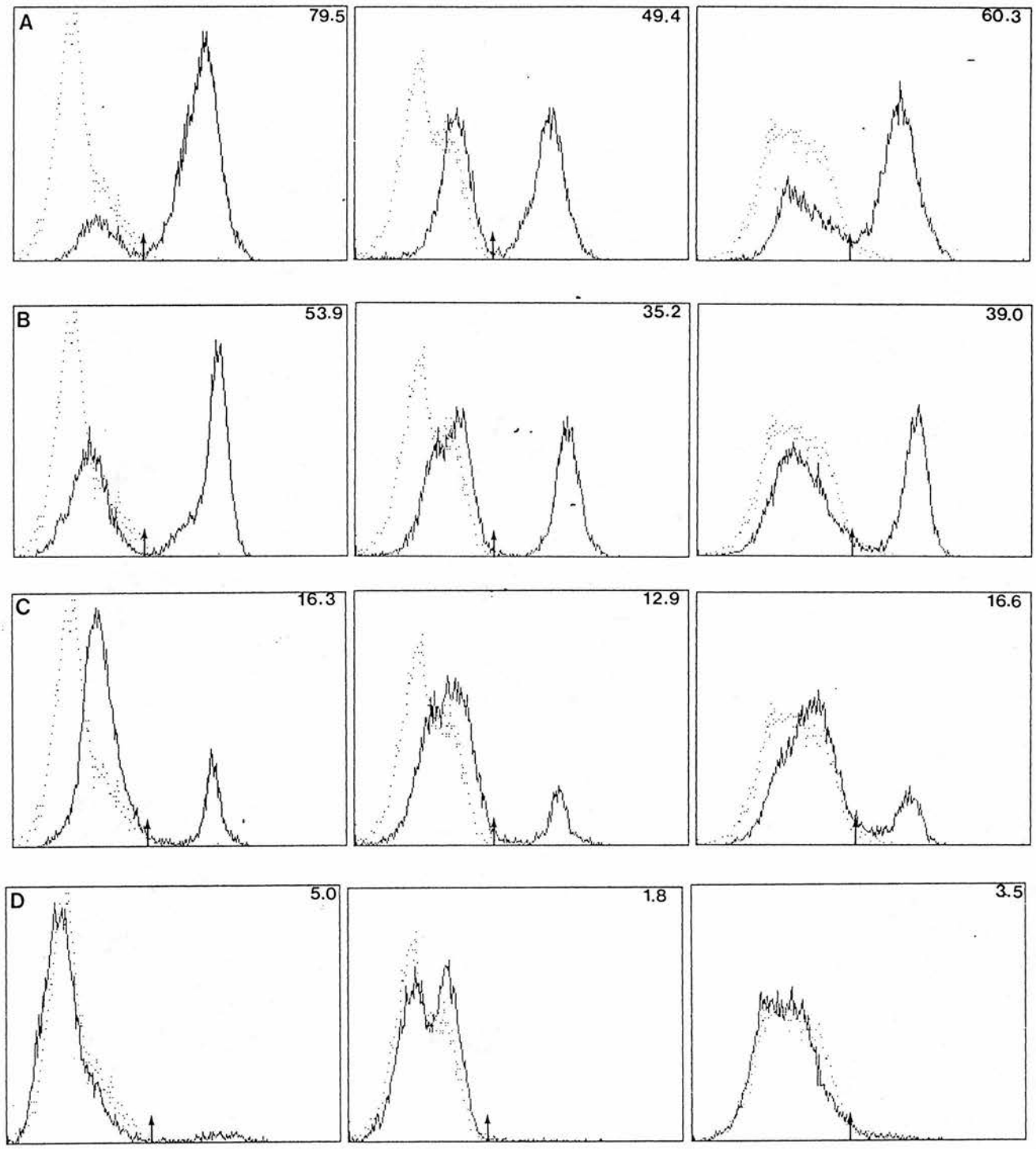
(B) $CD4^+$ cells showing a marked decrease nine days after infection.

(C) $CD8^+$ cells showing a moderate decrease nine days after infection.

(D) SBU-$T19^+$ T cells showing a moderate decrease nine days after infection.

The dotted profile in each case represents a negative cell sample while arrows indicate the distinction between negative and positive cells.

7.4


A
79.5
49.4
60.3
B
53.9
35.2
39.0
C
16.3
12.9
16.6
D
5.0
1.8
3.5
2
9
20
Days after infection

decline in the number of these cells was observed three to four days p.i. with a slight increase in output observed seven days p.i. The output of these cell then declined to even below preinfection levels as the skin reaction regressed.

7.3.4 Alterations in B lymphocyte kinetics

Prior to infection, proportions of efferent lymph cells expressing markers for B cells, surface immunoglobulin (SIg) and CD45R were low. SIg^+ cells comprised between 6.1 to 18.7%, while $CD45R^+$ cells comprised between 5.3 and 25.5% of efferent lymphocytes. In terms of absolute values, the output of SIg^+ cells from the resting node was between 0.12 and 2.14 x 10^7 per hour, while for $CD45R^+$ cells it was between 0.21 and 2.56 x 10^7 cells per hour. Following infection however, a dramatic increase in both the proportion and absolute cell output of both SIg^+ and $CD45R^+$ cells was observed (Figure 7.5). The initial increase occurred six to nine days p.i. when the proportions of SIg^+ and $CD45R^+$ cells rose to peaks of between 26 to 78% and 26 to 68% respectively in sheep 749 and 758. This was initially followed by a slight decline but a second increase was observed 13 to 16 days p.i. The increase in proportion of these cell populations was associated with an absolute increase in cell output. An eight to ten-fold increase in the absolute numbers of B cells was recorded. The output of SIg^+ cells on day eight p.i. was of the order of 10.4 to 20.3 x 10^7 per hour and by 12 to 15 days p.i., 8.6 to 14.7 x 10^7 per hour in the two sheep (749 and 758). Similar increases were observed for $CD45R^+$ cells whose output rose up to 10.3 to 16.1 x 10^7 per hour by day eight and 6.8 to 9.9 x 10^7 cells per hour between 12 to 14 days p.i. in sheep 749 and 758. With the regression of the local skin reaction, the proportions and output of these cells declined gradually but remained above pre-infection values.

7.3.5 Alterations in MHC Class I and II^+ $CD45^+$ cells

Prior to infection 11.1 to 15.4% of efferent lymph cells expressed MHC Class II antigens as revealed by labelling with anti-MHC II MAb (anti SBU-11). The output of MHC Class II^+ cells from the resting node was at the rate of 0.38 to 1.81 x

FIGURE 7.5 Representative flow cytometry profiles of efferent lymphocytes from prefemoral lymph node draining local skin reactions in sheep 758, two, nine and 20 days after infection with *T. congolense* TREU 1457. The proportion of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

(A) $CD1^+$ cells showing a marked increase nine days after infection.

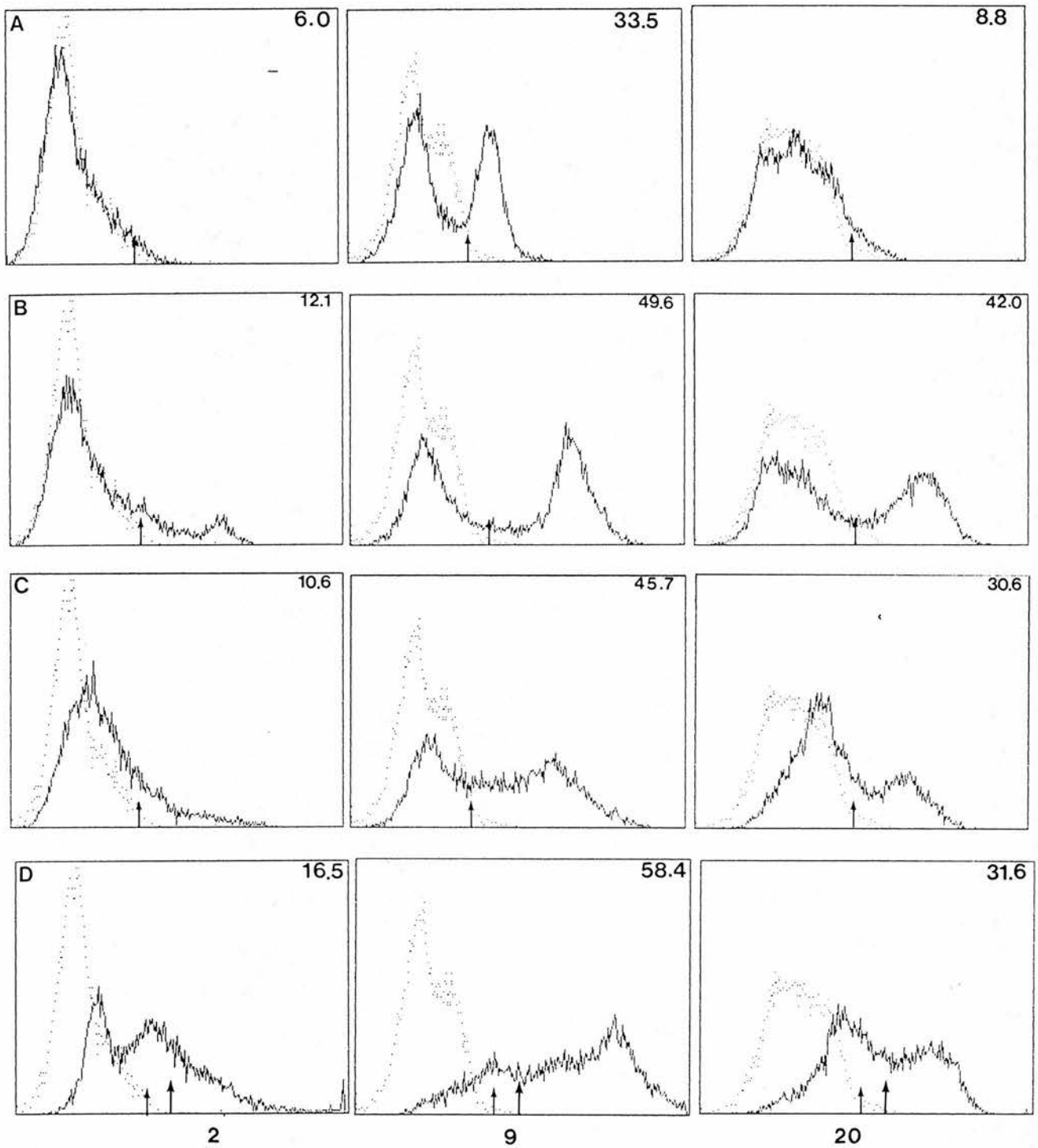
(B) $CD45R^+$ cells showing marked increase nine and 20 days after infection.

(C) MHC Class II^+ cells showing a marked increase nine and 20 days after infection.

(D) SIg^+ cells showing a marked increase nine and 20 days after infection.

The dotted profile in each case represents a negative control cell sample while arrows indicate the distinction between negative and positive cells.

7.5



Days after infection

10^7 per hour. From day one six after infection, the proportion of MHC Class II^+ cells increased (27.0 to 60 x 10^7 per hour) in the two sheep. During this period the rate of output of these cells was of the order of 8.8 to 12.7 x 10^7 per hour. A second phase of increase in MHC Class II^+ cells was observed 12 to 14 days p.i. when up to 5.8 to 10.8 x 10^7 per hour left the lymph node (Figure 7.6). The rate of cell output but not the proportions declined thereafter to nearly pre-infection levels by 21 days p.i. in sheep 758.

Proportions of cells expressing MHC Class I and leucocyte common antigens (LCA, CD45) remained well above 95% of efferent lymph cells throughout the course of infection. Therefore the rate of output of these cellular phenotypes increased with that of total cell output.

7.3.6 Phenotypes of cells expressing MHC Class II antigens

Prior to infection, most B cells (SIg^+ and $CD45R^+$) and some T cells expressed MHC Class II antigens. Following infection however, dual colour staining revealed that the increase in MHC Class II^+ cells was due to a larger increase in the number of B cells. The percentage of SIg^+ and $CD45R^+$ cells expressing MHC II^+ antigens increased following infection (Figure 7.7). Analysis of lymphoblasts during this period revealed that 50 to 73% were MHC Class II, 55 to 82% $CD45R^+$ cells and 60 to 87.1% SIg^+ cells. No major changes were observed in the proportions of small lymphocytes.

7.3.7 Effect of superinfection with an heterologous *T. congolense* serodeme (TREU 1881) on cellular responses in efferent lymph

At the time of inoculation with *T. congolense* TREU 1881, small numbers of trypanosomes were observed in the lymph. However, no trypanosomes were seen in the two days following challenge. From three days post-challenge low numbers of trypanosomes were present in the lymph (Figure E 4.4 Appendix IV).

No major alterations in cellular or lymphoblast output were detected in efferent lymph. Most T cell subsets showed little alterations in proportions except for $SBU\text{-}T19^+$ cells which declined between two and four days post-challenge. However,

FIGURE 7.6 Two colour immunofluorescence analysis of efferent lymphocytes from prefemoral lymph nodes draining local skin reactions in sheep 758 infected with *T. congolense* TREU 1457. Representative contour plots of cells collected two, nine and 20 days after infection stained for T lymphocyte subpopulations and MHC Class II. T lymphocytes were labelled red with phycoerythrin conjugated rabbit anti-mouse IgG. MHC Class II$^+$ cells were labelled green with FITC conjugated SW73.2 The double staining cells are in the upper right quadrant.

(A) Control cells.

(B) CD5$^+$ cells.

(C) CD4$^+$ cells.

(D) CD8$^+$ cells.

(E) SBU-T19$^+$ T cells.

7.6

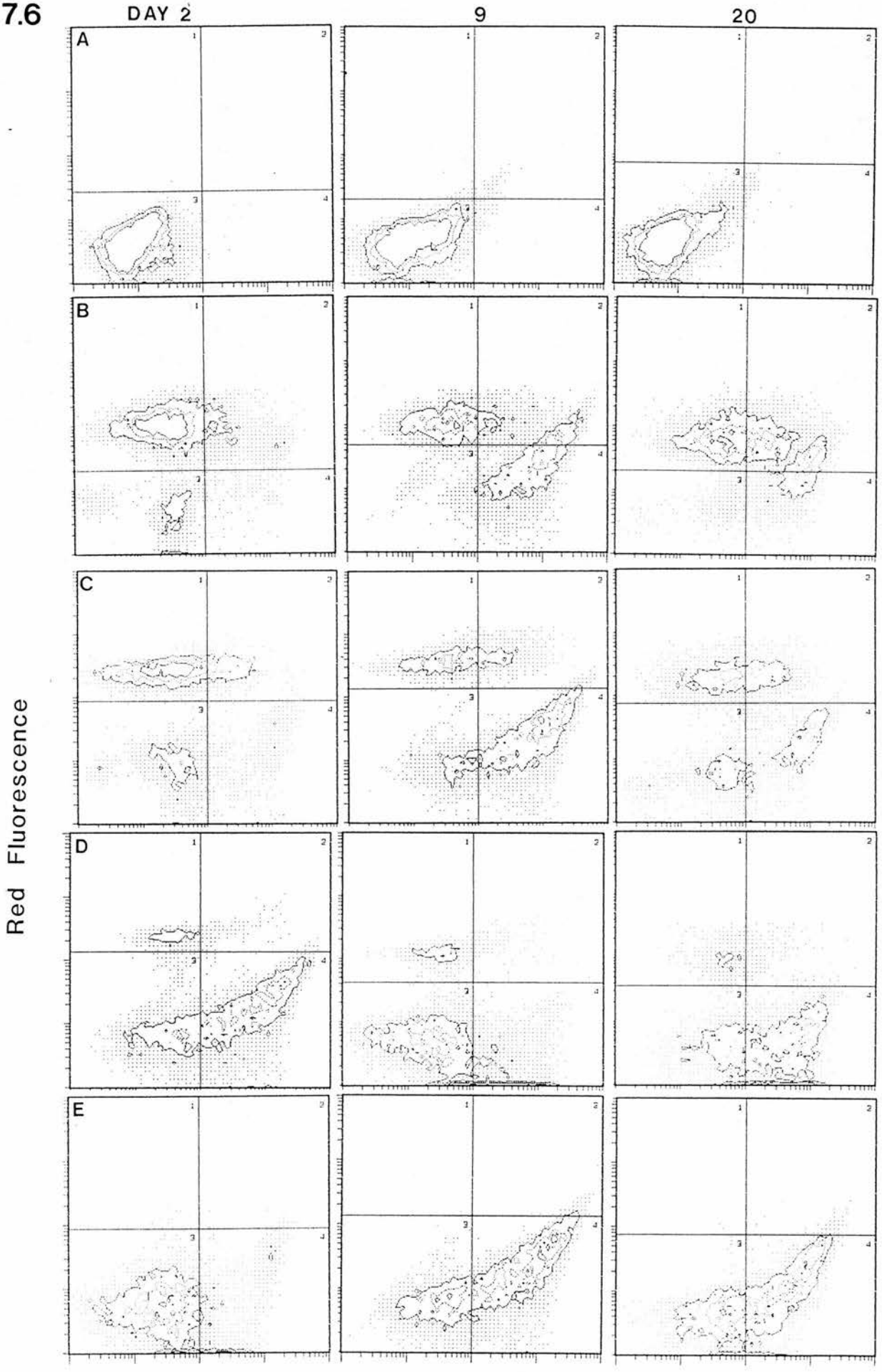
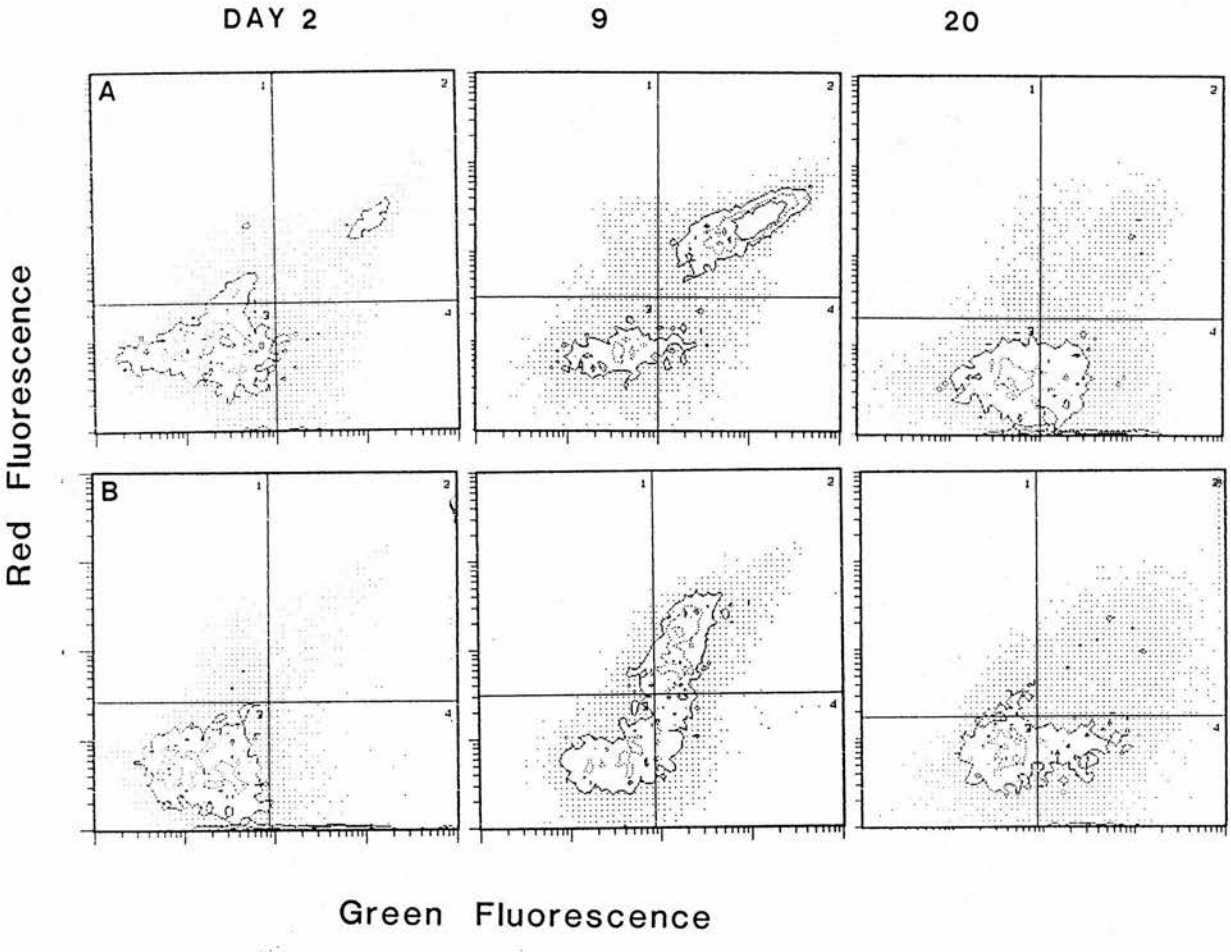


FIGURE 7.7 Two colour immunofluorescence analysis of efferent lymphocytes from prefemoral lymph node draining local skin reactions in sheep 758 infected with *T. congolense* TREU 1457. Representative contour plots of cells collected two, nine and 20 days after infection stained for $CD45R^+$ or SIg^+ lymphocytes and MHC Class II. Double staining cells are in the upper right quadrant.

(A) $CD45R^+$/MHC Class II^+ cells. RPE labelled $CD45R^+$ cells (red fluorescence) and SW73.2-FITC labelled MHC Class II^+ cells (green fluorescence).

(B) SIg^+/MHC Class II^+ cells. RPE labelled MHC Class II^+ cells (red fluorescence) and DAS-FITC Ig labelled SIg^+ cells (green fluorescence).

7.7



from the day of superinfection a gradual decline in absolute output of all T cell subsets ($CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$) was observed. Similarly, no major alterations in MHC Class II^+, SIg^+ and $CD45R^+$ cells, was observed although absolute numbers declined over time (Tables E 4.13 to 4.15 Appendix IV).

7.4 Discussion

The development of local skin reactions in sheep following intradermal inoculation with metacyclic *T. congolense* was accompanied by a lymph node enlargement and marked changes in the cellular output in efferent lymph. These changes were similar to those observed in efferent lymph of cattle and goats infected with *T. congolense* and in goats infected with *T. vivax* or *T. brucei* (Emery *et al.*, 1980; Barry and Emery, 1984; Akol and Murray, 1986; Dwinger *et al.*, 1990). The appearance of trypanosomes in the efferent lymph reaffirms suggestions that the lymphatic system is the major route for dissemination of the parasites from the skin (Luckins and Gray, 1979; Gray and Luckins, 1980).

T. congolense appears to form a secondary focus of proliferation in the draining lymph node (Luckins and Gray, 1979; Dwinger *et al.*, 1990) and trypanosomes therefore persisted in efferent lymph even up to 21 days p.i. Their presence cannot be accounted for by recirculation of the parasites from blood to lymph since *T. congolense* does not appear to recirculate through lymphatic system or invade tissues in large numbers once in the bloodstream (Akol and Murray, 1986). For example, in cattle infected with *T. congolense*, trypanosomes could be demonstrated in cannulated efferent lymphatics on the flank contralateral to that draining local skin reactions only by subinoculation into mice. Further evidence for the absence of a recirculating pool of trypanosomes from blood to lymph was shown by the presence of only six parasites in 1500 ml of efferent lymph collected over a period of 10 days from sheep infected by intravenous inoculation with bloodstream forms (Tizard *et al.*, 1978). In contrast *T. brucei* (Losos and Ikede, 1972) and *T. vivax* (Van den Ingh *et al.*, 1977; Murray *et al.*, 1980; Emery and Moloo, 1981) have the

capacity to leave the circulation, invade tissues and enter central lymphatics in large numbers (Emery *et al.*, 1980).

The response of draining lymph nodes to the presence of trypanosomes is reflected by the marked increase in cell output from the cannulated efferent lymphatic duct. This response is probably due to the cellular proliferation occurring within the draining lymph node during this period (Murray *et al.*, 1980). In the present study the cellular output observed was bimodal with the initial response coinciding with the onset of parasitosis and development of the chancre. The second phase of the response was not associated with any increase in the level of parasitosis in the lymph indicating that other contributory factor(s) were responsible. Since lymphocytes from peripheral lymph nodes and those from sites of chronic inflammation are known to recirculate preferentially from the blood into these sites (Chin and Hay, 1980; Isseukutz *et al.*, 1982), it is possible that lymphocytes from lymph nodes draining local skin reaction recirculated back into the same node in large numbers. However, this appears unlikely since most of these cells were removed through the cannulated lymphatic duct. The increase in cell output might also be due to cells emigrating from the local skin reactions, since it coincided with the regression of the skin lesion. During the development of trypanosomes in the local skin reaction and migration through the lymphatic system, new VATs emerge (Barry and Emery, 1984; Luckins *et al.*, 1990). Preliminary results have also shown that the proportion of metacyclic VATs in afferent lymph decreases after 10 days p.i. while no M-VATs were present in efferent lymph from seven days p.i. (Sutherland *et al.*, unpublished). The second response might therefore be due to the emergent VAT populations.

Marked alterations were observed in proportions and absolute output of various lymphocyte subpopulations in efferent lymph. The most characteristic change was an increase in proportion of B cells and a decrease in T cell subpopulations. However, considering the increase in total cell output, the absolute output of T cells increased two-fold compared with a ten-fold increase in B cell output. Decreases in

proportions of T lymphocyte subpopulations were therefore relative to an increase in proportion of B cells. However, the absolute output of SBU-T19$^+$ did not appear to change although the decline in its proportion was most marked. This T cell subpopulation appears not to participate in most inflammatory and immunological reactions (Meeusen *et al.*, 1989).

B cells comprise more than 82% of the increased proportion of lymphoblasts in efferent lymph. The products of this intense B cell proliferation might be responsible for production of neutralizing antibodies against metacyclic trypanosomes which are demonstrable in efferent lymph from 10 days after infection (Akol and Murray, 1986; Dwinger *et al.*, 1990; Luckins *et al.*, 1990). The increase in proportion and absolute output of MHC Class II$^+$ cells corresponded with the increase in B cells. Dual colour staining confirmed that most of MHC Class II$^+$ cells were indeed SIg$^+$ or CD45R$^+$ (B cells). However, some of the MHC Class II$^+$ cells were T cells indicating a degree of activation of T cell subpopulations (Hopkins *et al.*, 1986). The majority of these T cells were CD5$^+$ and CD4$^+$ cells. Activated T helper (CD4$^+$) cells are crucial in providing a key stimulus leading to B cell proliferation and production of antibodies (Kindred and Shreffler, 1972; Tite, Kaye and Jones, 1984; Lanzavecchia, 1985). In experimental leishmaniasis in mice, B cells of infected mice are polyclonally stimulated to proliferate and produce antibodies by T-helper cells (L3T4$^+$) obtained from lymph nodes draining lesions of *Leishmania major* infected mice (Lohoff, Matzner and Röllinghoff, 1988). A major feature of such polyclonal B cell stimulation is the increased number of B cells. It is possible that similar B cell stimulation occurs in lymph node draining local skin reactions.

Several mechanisms could account for alterations in proportions and absolute numbers of various lymphocyte subpopulations in efferent lymph during the course of development of local skin reaction. Lymphocytes continuously migrate from blood to lymph in the lymph node and these cells account for more than 95% of cellular output in efferent lymph, even during an immune response (Hall and Morris, 1963, 1965;

Morris, 1968). Increased blood flow and migration rate of lymphocytes into the lymph node undergoing an inflammatory or immune response might account for the increased cellular output, (Hall and Morris, 1963, 1965). However, the disproportionate increase in B and T cell output is not easily explained. It is possible that both T and B cells migrate into the lymph node at the same rate, but while T cells are retained within the node, B cells after clonal selection, proliferate at a higher rate in the lymph node before appearing in the efferent lymph. This B cell proliferation could partly be brought about by non-specific stimulation by *T. congolense* derived mitogenic factors (Assoku, Tizard and Nielsen, 1977; Assoku and Tizard, 1978; Tizard *et al.*, 1978). The alterations of lymphocyte subpopulations in efferent lymph is bound to have effects on the immune response. The lymph node plays an important role in recruiting and providing an environment suitable for cell interactions, selection and proliferation of antigen-specific cells prior to dissemination to the systemic circulation. This response is crucial in establishing a more effective immune response against tsetse transmitted trypanosomiasis and might account for the differences in host susceptability between Zebu and N'dama cattle and various wild ruminants where resistance appears to be associated with relative effectiveness of the immune response during the establishment of infection (Dwinger, 1985; Akol *et al.*, 1986).

CHAPTER EIGHT

HAEMATOLOGICAL CHANGES AND ALTERATIONS IN PERIPHERAL BLOOD LEUCOCYTE PHENOTYPES DURING THE EARLY STAGES OF *T. CONGOLENSE* INFECTION IN SHEEP

8.1 Introduction

Peripheral blood is a traffic route for lymphocytes which continuously enter and exit lymphoid and non-lymphoid organs including inflammatory lesions (Westermann and Pabst, 1990). The determination of lymphocyte subset dynamics could therefore provide information on the lymphoid response in general. Variations in peripheral blood lymphocyte subpopulations have been found in human patients suffering from leishmaniasis, measles, leprosy and schistosomiasis (Bach *et al.*, 1981; Joffe, Sukha and Rabson, 1983; Feldmeier *et al.*, 1985; Jaroskova *et al.*, 1986). In domestic animals, variations in peripheral blood lymphocyte subpopulations have been observed in border disease and bovine leukemia induced lymphosarcoma in sheep (Gatei *et al.*, 1990; Woldehiwet and Sharma, 1990) and neoplastic conditions in cattle (Sulochana *et al.*, 1985).

Haematological alterations of both red and white blood cells are major pathophysiological features of African trypanosomiasis in domestic ruminants (Wellde *et al.*, 1974; Fiennes, Jones and Laws, 1976; Valli, Forsberg and Mills, 1979; Valli and Mills, 1980; Ellis *et al.*, 1987). Studies carried out in cattle infected with *T. congolense* show that initially the response is characterized by a lymphocytopaenia followed by a lymphocytosis after the onset of parasitaemia (Valli *et al.*, 1979; Ellis *et al.*, 1987). These changes are associated with a decrease in various lymphocyte subpopulations followed by a progressive increase in B lymphocytes and steady decline in T cell subpopulations as the infection progresses (Ellis *et al.*, 1987).

Since infection with *T. congolense* in sheep is associated with changes in lymphocyte subpopulations in the skin, afferent lymph, draining lymph nodes and efferent lymph, it is possible that these changes are reflected in similar alterations in cells in the peripheral blood.

In order to investigate if, indeed, these changes were evident peripheral blood leucocytes from *T. congolense*-infected sheep at both early and late stages of infection were analyzed to determine the extent of phenotypic variation in lymphocytes and its

association with the response already characterized in the skin, lymph nodes and draining lymphatics.

8.2 Materials and Methods

8.2.1 Trypanosomes and infection

Sheep were infected with 2 x 10^5 culture-derived metacyclic forms of *T. congolense* TREU 1457 inoculated intradermally.

Details of the experimental design are given in Table 8.1. Two separate experiments were carried out. The first study (Experiment 1) was carried out prior to availability of a flow cytometer and the cells were analyzed by fluorescence microscopy. Experiment 2 was carried out on a further four sheep and cells analyzed by flow cytometry.

8.2.2 Clinical, haematological and parasitological parameters

The measurements of clinical, haematological and parasitological parameters were carried out as described in Section 3.3 and 3.4. Total white blood cell (TWBC) counts and red blood cell counts (RBC) were determined on a Coulter Counter (Coulter Electronics Ltd) as decribed in Section 3.3. Differential leucocyte counts were made on 200 leucocytes on each Giemsa stained thin blood film. Total number of lymphocytes, neutrophils, monocytes and eosinophils were estimated by multiplying TWBC by the percentage of each leucocyte type.

8.2.3 Monoclonal antibodies, immunofluorescent staining and analysis of cells

Peripheral blood leucocyte preparations, MAbs used, immunofluorescent staining and analysis are described in Section 3.4 and 3.7. Absolute number of cells per ml of blood were estimated by multiplying the percentage of cells reactive for each MAb by the number of total mononuclear leucocytes per ml of peripheral blood.

8.3 Results

8.3.1 Development of local skin reactions and onset of parasitaemia

Results are summarized in Table 8.2. In Experiment 1, all four sheep developed local skin reactions by day five after infection. These reactions reached

TABLE 8.1 Experimental design

Experiment	Number of animals	Method of PBL preparation	Day of treatment	Method of analysis of leucocyte subsets	Experimental period (days)
1	4 sheep (101,103,104,105)	Density centrifugation of defibrinated blood	61	Fluorescence microscopy	79
2	4 sheep (940,941,942,943)	Tris-ammonium chloride lysis of heparinised blood	38	Flow cytometry	69

TABLE 8.2 Development of local skin reactions and onset of parasitaemia in sheep following infection with *T. congolense* TREU 1457

	Sheep Number	Local skin reactions Days of detection	Peak	Onset of parasitaemia
Expt 1				
	101	5	7	19
	103	4	7	15
	104	5	8	20
	105	5	7	20
Expt 2				
	940	-	-	27
	941	6	8	13
	942	-	-	-
	943	7	9	20

their peak by day seven and then regressed. Parasitaemia was detectable in these sheep by microhaematocrit method between 15 to 20 days (Figure 8.1). In the second experiment only two sheep (941 and 943) developed detectable local skin reactions on day six and seven respectively which reached a peak on days eight and nine. These two sheep developed parasitaemia on days 13 and 20 after infection respectively. However, one sheep (940) which did not develop a local skin reaction became parasitaemic by day 27 after infection. Sheep 942 neither developed a local skin rection nor were parasites detected even by mice inoculation and will not therefore be referred to in the description of the results.

8.3.2 Clinical, parasitological and haematological changes

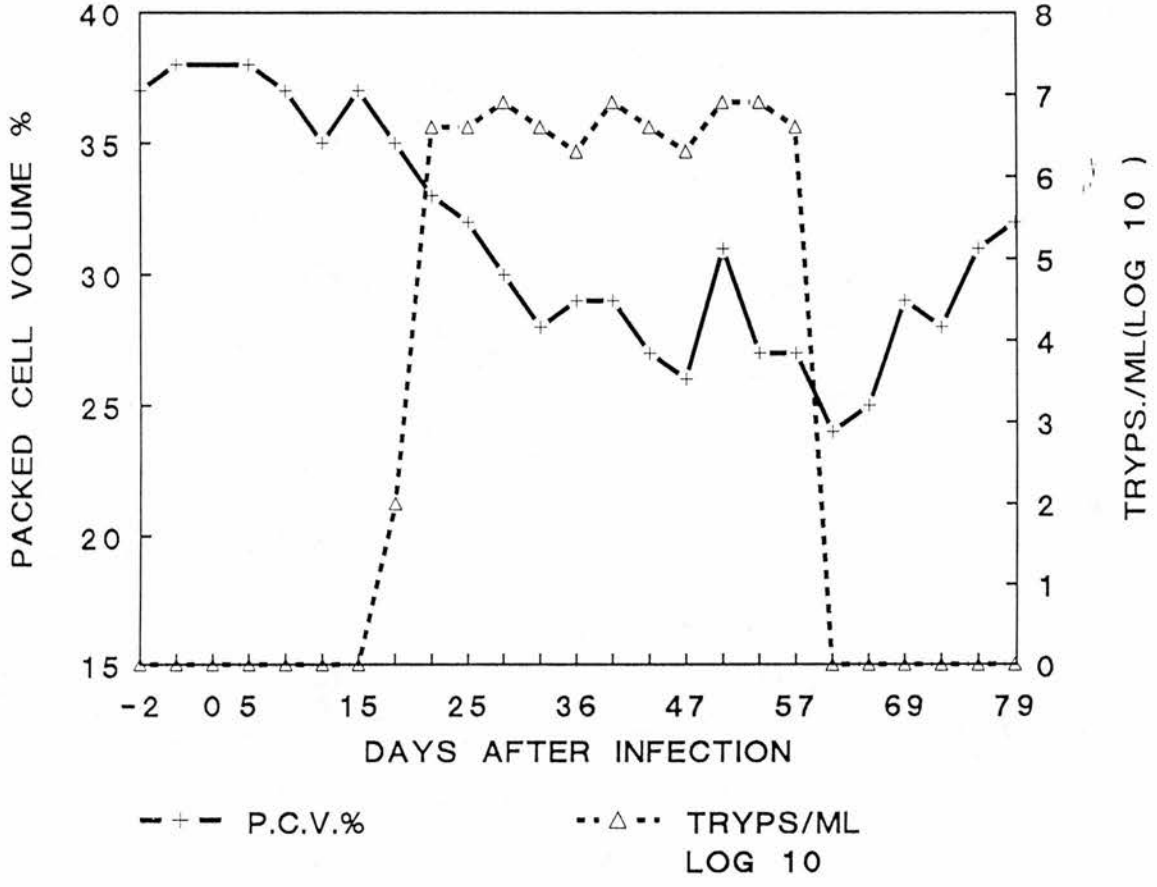
In the first experiment, the packed cell volume (PCV) of the four sheep declined gradually after the onset of parasitaemia (Figure 8.1; Appendix V) from a mean pre-infection value of 39% to below 30% 29 days post-infection. Changes in TWBC and differential leucocyte counts are shown in Figure 8.2 and in Appendix V. Three of the sheep (101, 103, 105) showed a gradual increase in TWBC and lymphocytes after the onset of parasitaemia. This increase was variable and lasted throughout the course of infection. In sheep 104, an increase in TWBC and lymphocytes was observed from five days with the peak on 12 days p.i. when it was five times pre-infection values. This gradually declined to pre-infection levels by day 21 p.i. only to increase again 47 days p.i. In all the sheep, no major changes were observed in the levels of monocytes, neutrophils or eosinophils.

In the second experiment, sheep were monitored for up to 38 days p.i. The onset of parasitaemia was much later than in the first experiment. PCV levels did not change appreciably in the sheep which became parasitaemic. Only in sheep 941 which became parasitaemic 13 days p.i. did the PCV decline to below 25% from a pre-infection value of 29%. Following treatment however, the PCV rose above pre-infection value within four weeks (Figure 8.3). There was no overall trend in changes in TWBC or different leucocyte types (Figure 8.3). An initial decline in TWBC and

FIGURE 8.1 Representative example of changes in packed cell volume (PCV %) and parasitaemia (log_{10} trypanosomes per ml) in peripheral blood of a sheep 101 infected with *T. congolense* TREU 1457.

FIGURE 8.2 Representative example of the sequential analysis of total numbers of white blood cells (TWBC), lymphocytes, neutrophils, monocytes and eosinophils per ml of blood of sheep 101 during the course of infection with *T. congolense* TREU 1457.

8.1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35
30
25
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15
8
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5
4
3
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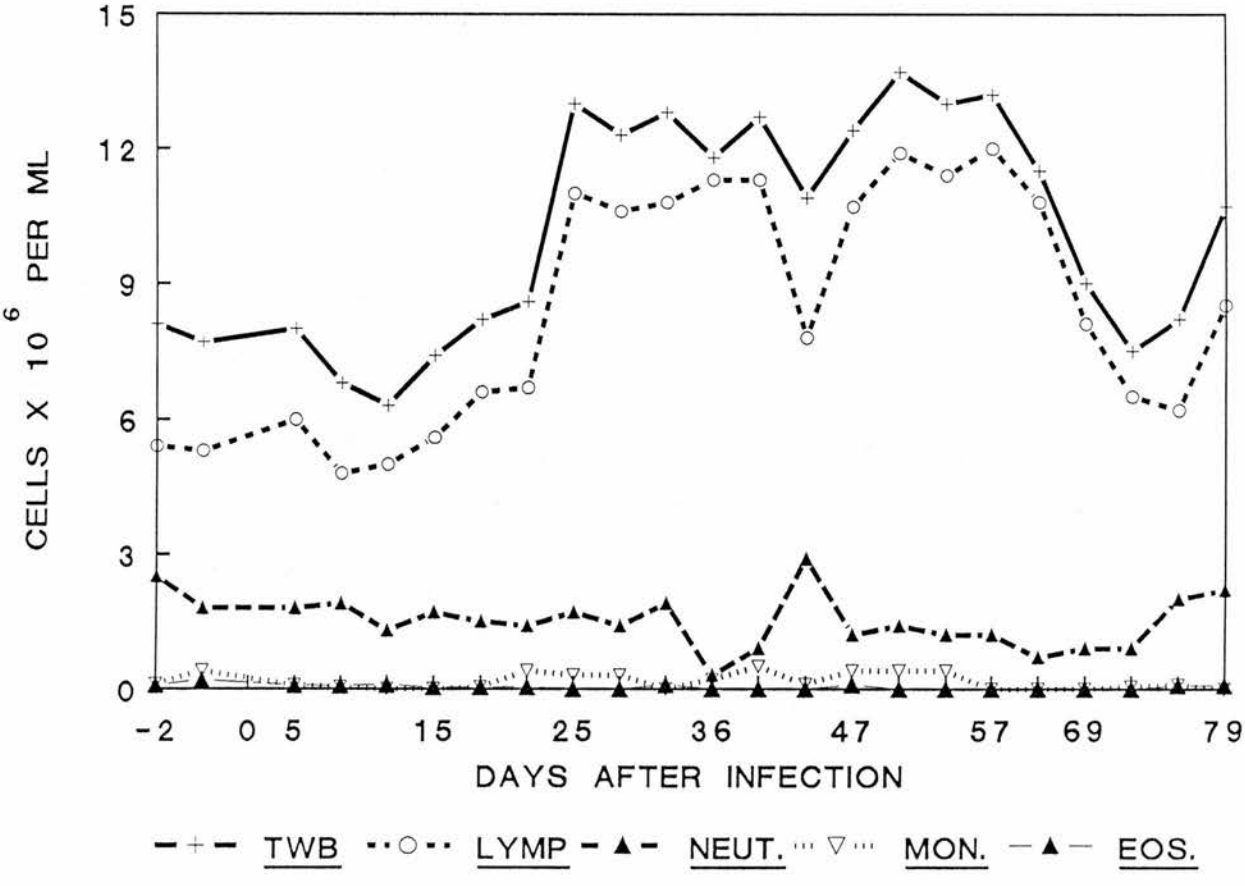
0
-2 0 5 15 25 36 47 57 69 79
DAYS AFTER INFECTION
P.C.V.%
TRYPS/ML
LOG 10

8.2


CELLS X 10 6 PER ML
15
12
9
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DAYS AFTER INFECTION
TWB
LYMP
NEUT.
MON.
EOS.

FIGURE 8.3 Representative example of changes in a) packed cell volume (%) and parasitaemia, b) leucocytes, c) T cell subpopulations and d) SIg^+ and MHC Class II^+ cells in peripheral blood of sheep 940 following infection with *T. congolense* TREU 1457.

Legends

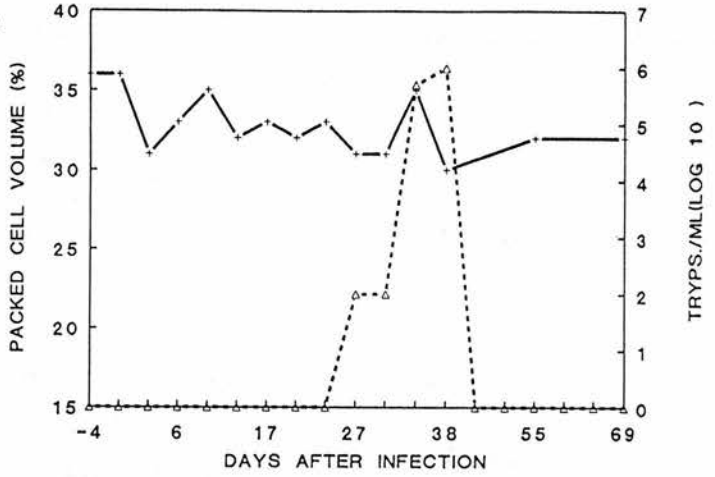
(A) Packed cell volume (%) –+–
Parasitaemia ··△··

(B) Total white blood cells –+–
Lymphocytes ··○··

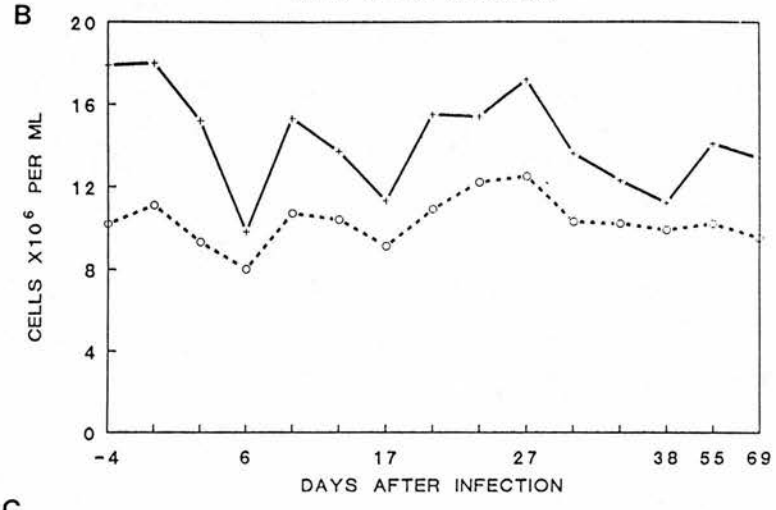
(C) $CD5^+$ cells –+–
$CD4^+$ cells ··○··
$CD8^+$ cells – ○–

(D) SIg^+ cells ··●··
MHC Class II^+ cells –▲–

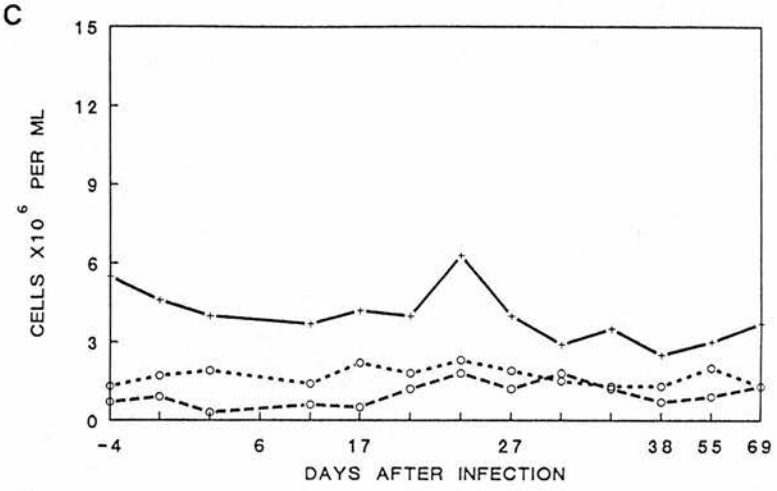
8.3 A


PACKED CELL VOLUME (%)
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION

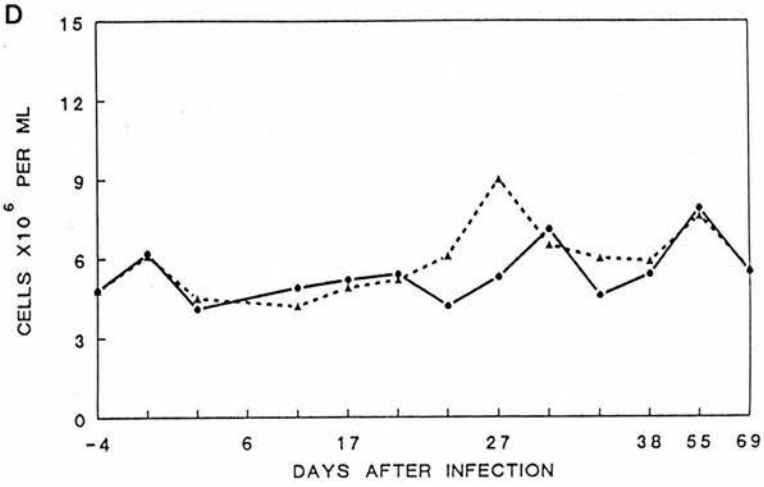
B


CELLS X10⁶ PER ML
DAYS AFTER INFECTION

C


CELLS X10⁶ PER ML
DAYS AFTER INFECTION

D


CELLS X10⁶ PER ML
DAYS AFTER INFECTION

lymphocytes was observed in sheep 940 and 943 following infection and prior to development of parasitaemia. TWBC and lymphocyte levels in sheep 941 remained low until after trypanocidal therapy when an increase was observed within two weeks. The levels of monocytes, neutrophils and eosinophils did not show any major changes during infection.

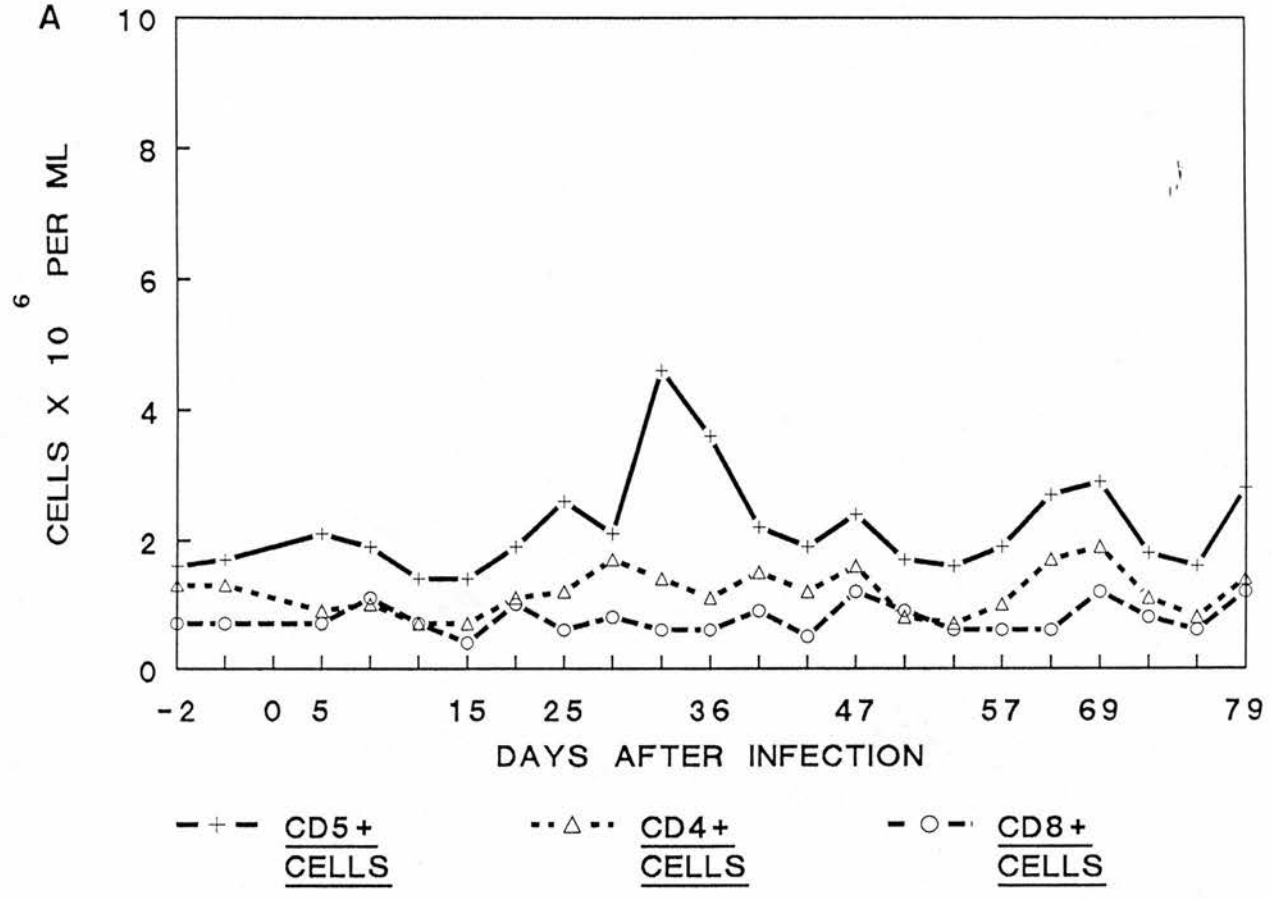
8.3.3 Leucocyte subpopulation dynamics

In the first experiment, changes in PBM subpopulations analyzed by fluorescence microscopy are illustrated in Figures 8.4. Among T lymphocyte subsets there was a general decline in the number of $CD5^+$ cells following infection and up to the time that trypanosomes appeared (Figure 8.5). In three of the sheep (101, 103, 105) from 25 days p.i. there was a variable increase in $CD5^+$ cells. A rise in $CD5^+$ cells only occurred in sheep 104 after trypanocidal drug therapy. The numbers of $CD4^+$ cells declined following infection and in two of the sheep (103 and 105) an increase in their number was observed from 47 days p.i. The most notable changes were observed with $CD8^+$ cells. The numbers of these cells declined (notably sheep 103, 104, 105) following development of parasitaemia and only returned to pre-infection levels in sheep 103 and 105 47 days p.i.

Analysis of PBM with MAb defining MHC Class II^+ cells and polyclonal sera identifying SIg^+ (B) cells revealed a marked alteration in these populations (Figure 8.4). A steady increase in the proportions and numbers of SIg^+ and MHC Class II^+ cells was observed in all four sheep from 11 to 18 days p.i. coinciding with the onset of parasitaemia. This increase was up to four times the pre-infection values at times.

In the second experiment, changes in PBM subpopulations analyzed using a flow cytometer is shown in Figure 8.3 while representative FACS profiles are given in Figure 8.5. There was little change in the proportions of $CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$ cells. In two of the sheep (940 and 941), absolute values of $CD5^+$ cells decreased following infection. However, in one of the sheep (943), an initial increase in these cells from day three to day 10 p.i. was followed by a decline. No major

FIGURE 8.4 Sequential analysis of changes in the total number of a) T cell subpopulations $CD5^+$, $CD4^+$ and $CD8^+$ cells and b) SIg^+ and MHC Class II^+ cells per ml of peripheral blood of sheep 101 during the course of infection with *T. congolense* TREU 1457.


A
10
8
6
4
2
0
CELLS X 10 6 PER ML
-2 0 5 15 25 36 47 57 69 79
DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS

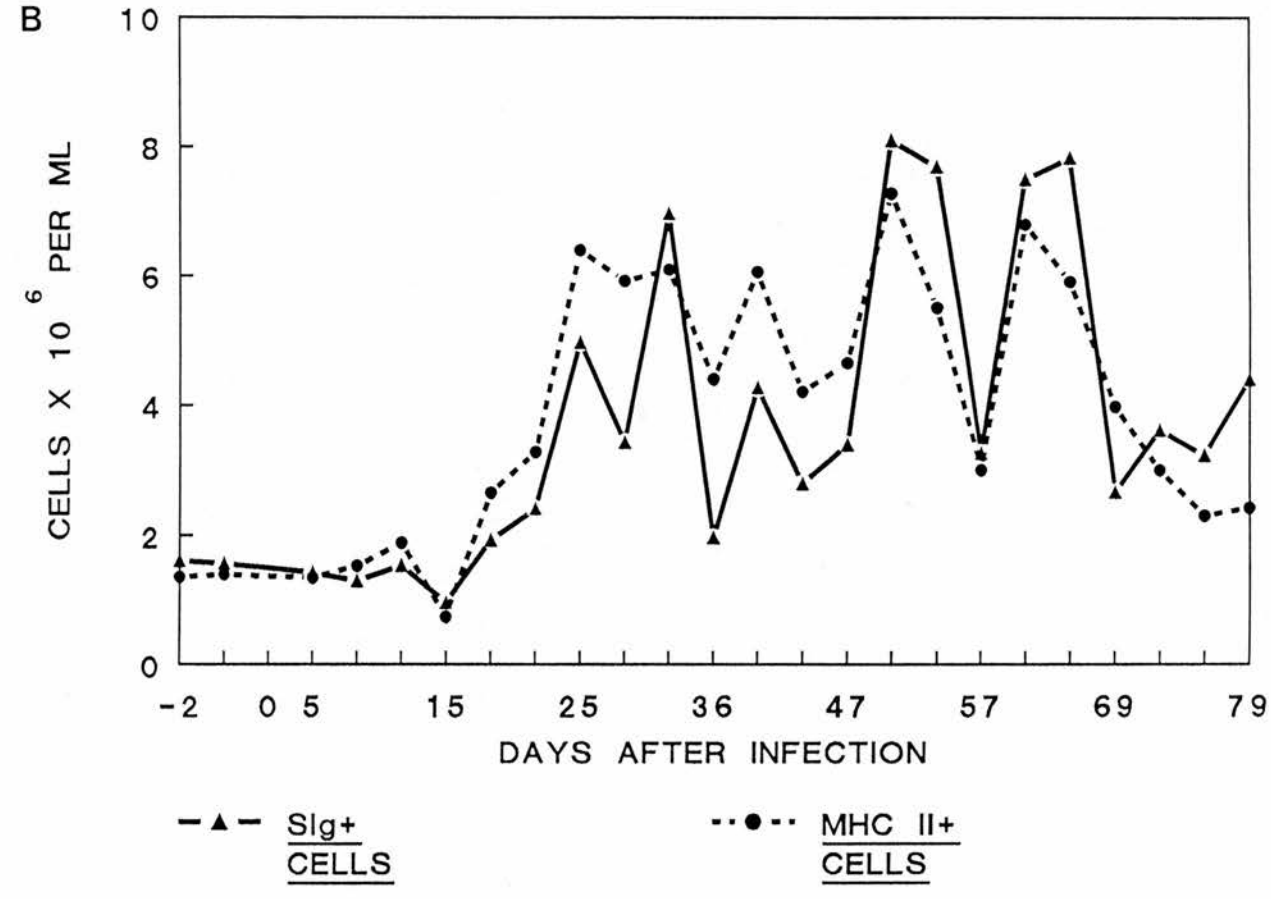

B
10
8
6
4
2
0
CELLS X 10 6 PER ML
-2 0 5 15 25 36 47 57 69 79
DAYS AFTER INFECTION
SIg+ CELLS
MHC II+ CELLS

FIGURE 8.5 Representative flow cytometry profiles of peripheral blood leucocyte subpopulations of sheep 940 collected on days -1, 20 and55 after infection with *T. congolense* TREU 1457. The proportions of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

(A) $CD5^+$ cells.

(B) $CD4^+$ cells.

(C) $CD8^+$ cells.

(D) SBU-$T19^+$ T cells.

The dotted profile in each case represents a negative cell sample while the arrows indicate the distinction between negative and positive cells.

8.5

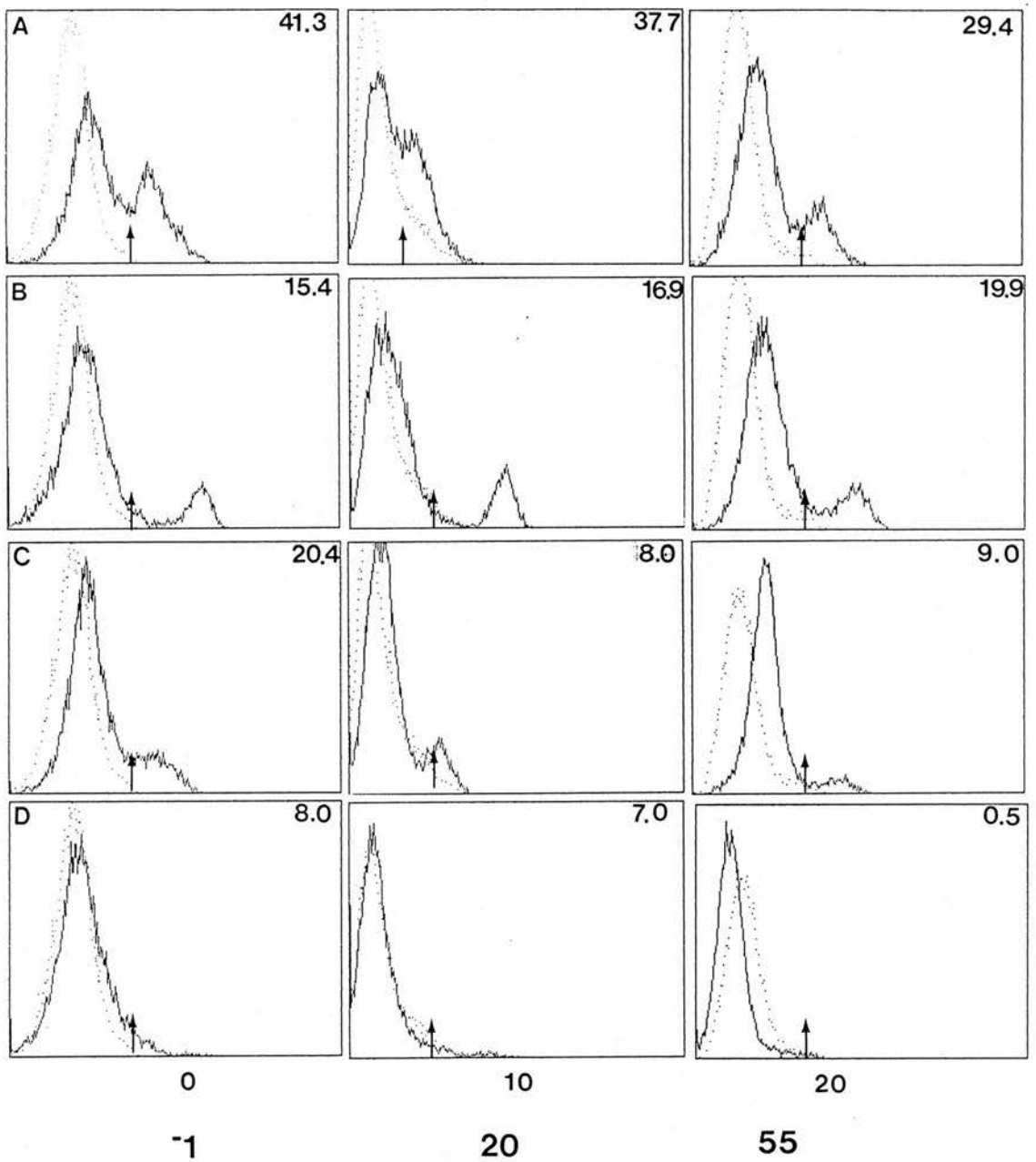

A
41.3
37.7
29.4
B
15.4
16.9
19.9
C
20.4
8.0
9.0
D
8.0
7.0
0.5
0
10
20
-1
20
55
Days after infection

FIGURE 8.5 Representative flow cytometry profiles of peripheral blood leucocyte subpopulations of sheep 940 collected on days -1, 20 and 55after infection with *T. congolense* TREU 1457. The proportions of each cell subpopulation is indicated as a percentage of 10,000 cells analyzed.

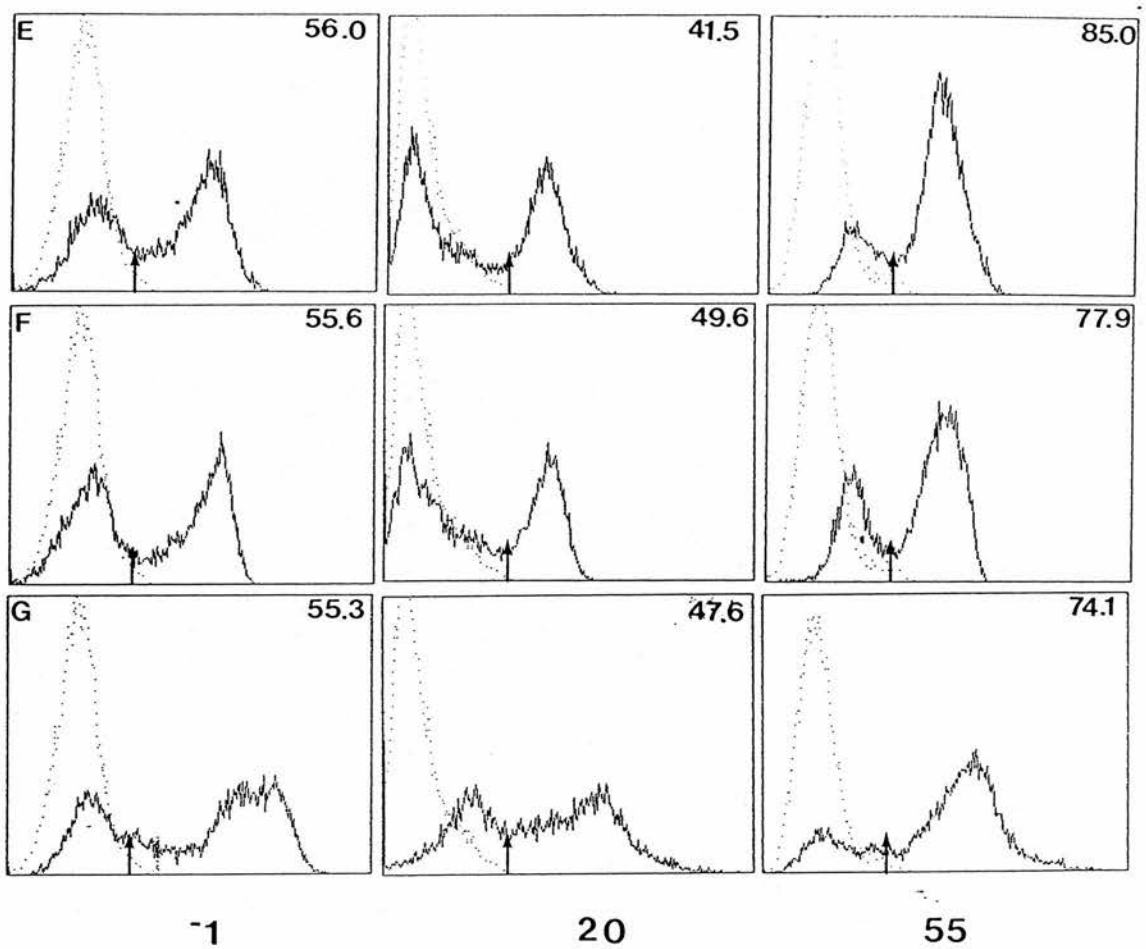
(E) $CD45R^+$ cells.

(F) MHC Class II^+ cells.

(G) SIg^+ cells.

The dotted profile in each case represents a negative cell sample while the arrows indicate the distinction between negative and positive cells.

8.5


E
56.0
41.5
85.0
F
55.6
49.6
77.9
G
55.3
47.6
74.1
-1
20
55
Days after infection

alterations was observed for $CD4^+$, $CD8^+$ or SBU-$T19^+$ cells except for a brief period of increase prior to development of parasitaemia.

The changes in MHC Class II^+ and SIg^+ cells in these sheep were not as marked as those observed in the first experiment. The proportions of these cells increased slightly following onset of parasitaemia. The absolute values of these cells in Sheep 941 declined over time and coincided with a decline in TWBC, and only increased following treatment. However in the other two sheep, a slight decline in numbers of these cells was followed by a fluctuating though slight increase after the onset of parasitaemia (Figure 8.5).

8.4 Discussion

All sheep showed changes in total leucocyte counts and alterations in the numbers of various lymphocyte subpopulations after onset of patent parasitaemia in blood. Trypanosomes were detected in peripheral blood from 13 days p.i., after the regression of local skin reaction. This is similar to observations made in cattle and goats following infection with *T. congolense* (Akol and Murray, 1986; Dwinger *et al.*, 1987). However, there was wide variation in the prepatent period especially in the second experiment as shown by lower overall levels of parasitaemia, non-infection of one sheep, and development of local skin reactions in only two of the sheep. This probably arose due to variation in virulence between the trypanosome inocula since these two groups of sheep were infected with different cultures of the same stock of *T. congolense*. The drop in PCV following onset of parasitaemia in *T. congolense* infected sheep was similar to that observed in cattle and goats infected with *T. congolense* (Akol and Murray, 1986; Dwinger *et al.*, 1989). However, the decline in PCV was closely associated with the level of parasitaemia. Thus in the sheep which developed low parasitaemia, the drop in PCV was not as pronounced as in animals with high parasitaemia (Experiment 2). A fall in PCV is an indicator of degree of anaemia and therefore the severity of infection. No major clinical symptoms except brief phases of pyrexia accompanied the onset of parasitaemia or drop in PCV. It is

therefore concluded that the sheep developed only a low grade and mild infection with culture derived metacyclic forms of *T. congolense* TREU 1457.

Despite the low pathogenicity of the trypanosomes, changes were still observed in total white blood cell counts (TWBC). In the first group of sheep the TWBC remained at pre-infection levels until after onset of parasitaemia when a leucocytic phase was observed. In the second group of sheep which developed low parasitaemia a leucopaenia was observed prior to and after onset of parasitaemia. Leucocytosis was observed only after trypanocidal therapy. Initial leucopaenia corresponding to the onset of anaemia has been well documented in cattle infected with syringe passaged *T. congolense* (Fiennes, Jones and Laws, 1946; Naylor, 1971; Wellde *et al.*, 1974; Ellis *et al.*, 1987). In chronic infections however, both persistent leucopaenia (Wellde *et al.*, 1974) and leucocytosis (Valli, Forsberg and Lumsden, 1978) have been reported in experimentally infected animals. In sheep infected with *T. congolense* the only variation accounting for the differences in the early responses was probably the virulence of the particular infecting trypanosome inocula. The changes in leucocyte levels were due to alterations in lymphocyte levels and not neutrophils or eosinophils. This is in agreement with studies carried out in *T. congolense* infected cattle (Ellis *et al.*, 1987) but was in contrast to previous studies documenting a neutropenic in *T. congolense* infected cattle (Wellde *et al.*, 1974; Valli *et al.*, 1979).

A marked feature observed in peripheral blood is the increase in both the proportion and absolute numbers of B cells. This was seen in infected sheep after 38 days p.i. Similar observations have been made in N'dama and Boran cattle infected with *T. congolense* (Ellis *et al.*, 1987). This increase in B cells is probably due to a marked lymphoid proliferation in lymph nodes draining local skin reactions and other centrally placed lymph nodes and the spleen of infected hosts involving mainly B cell areas (Valli and Forsberg, 1979; Morrison and Murray, 1979; Murray *et al.*, 1980) (Section 5.3 and 7.3). Similar profound B lymphocyte proliferation in lymph nodes,

bone marrow and spleen of mice infected with *T. congolense* has been observed (Mayor-Withey *et al.*, 1978; Morrison *et al.*, 1981; Morrison, Murray and Bovell, 1982b; Morrison, Murray and Hinson, 1982a). The increase in B cells in peripheral blood might be responsible for the increase in circulating IgM in infected animals (Luckins and Mehlitz, 1976; Masake *et al.*, 1983). Enhanced survival of *T. congolense* infected cattle has been linked to effective B cell lymphocytosis in trypanotolerant cattle (Labohm, 1982). Trypanotolerant N'dama cattle which are able to control parasitaemia, anaemia and weight loss, have significantly higher numbers of B cells than susceptible Boran cattle and show a persistent B cell lymphocytosis from 35 to 53 days after *T. congolense* infection (Ellis *et al.*, 1987). This is probably related to effective production of specific anti-trypanosomal antibodies.

The increase in MHC Class II^+ cells is probably a reflection of an increase in percentage and absolute values of circulating B cells since the two phenotypes showed a similar trend. In infected cattle a similar correlation between increases in B cells and MHC Class II^+ cells was observed (Ellis *et al.*, 1987). MHC Class II antigens are constitutively expressed on B cells, activated T cells and monocytes (Singer and Hodes, 1983; Hopkins *et al.*, 1986; Lalor *et al.*, 1986; Emery *et al.*, 1987) and can therefore be used to gauge the efficiency of host response to a parasite (Ellis *et al.*, 1987).

Alteration in T cell subpopulations especially $CD8^+$ cells in *T. congolense* infected sheep was characterized by a slight decline in proportions and absolute numbers from 25 days p.i. in the first experiment. Similar observations have been made in cattle infected with cyclically transmitted *T. congolense* (Ellis *et al.*, 1987). The cause of this selective depletion of T cell subpopulations is not clear although it could be related to the absolute increase in percentage of circulating B cells and an increased B cell output from lymph nodes draining local skin reactions (Section 7.3). T cell dependent areas of these lymph nodes were also depleted of small lymphocytes. Similarly, longstanding infections in cattle lead to depletion of cells from the

paracortical areas of lymph nodes (Morrison and Murray, 1979b). This might indicate that the decline in T cells is due to depletion in the lymphoid organs. Depletion of $CD8^+$ cells in rats using specific MAbs is associated with a decrease in growth of *T. brucei* and (Bakhiet *et al.*, 1990) probably due to increased antibody production. It is possible that in sheep infected with *T. congolense*, the decrease in $CD8^+$ cells results in a loss of suppressor cells and therefore an increase in the number of proliferating B cells. T helper cell deficiency might be responsible for the retardation in the switching from IgM to IgG synthesis by B cells resulting in high IgM levels observed in trypanosome infected animals (reviewed by Mansfield, 1978; Diggs *et al.*, 1988). These features are characteristic for immunodepression observed during trypanosome infections. T cells represent a major protective as well as regulatory cell system in mammals and a defect in one or more T cell subpopulations may have dramatic effects on the ability of an infected host to resist infections with other pathogens or to regulate its own immune system.

CHAPTER NINE

ATTACHMENT AND PHAGOCYTOSIS OF TRYPANOSOMES *IN VIVO* BY MACROPHAGE/DENDRITIC CELLS IN AFFERENT LYMPH DRAINING LOCAL SKIN REACTIONS

9.1 Introduction

Afferent lymph from the skin of sheep contains a mixture of cells including lymphocytes, macrophages and dendritic or 'veiled' cells which are in direct contact with antigens injected into the skin (Smith *et al.*, 1970). Macrophages and dendritic cells make up five to 20% of afferent cells (Smith *et al.*, 1970). These cells have been shown by electron microscopy and immunofluorescence to engulf immune complexes *in vivo*, when antigen is administered intradermally into immunized sheep (Hall and Robertson, 1984; Harkiss, Hopkins and McConnell, 1990).

Several investigations have demonstrated that, *in vitro,* the non-pathogenic trypanosomes *T. lewisi* and *T. musculi*, as well as pathogenic *T. brucei* and *T. gambiense* adhere to rat, rabbit and mouse peritoneal exudate cells and are eventually phagocytosed (Lange and Lysenko and Enriquez, 1960; Lumsden and Herbert, 1967; Takayanagi and Nakatake, 1974a,b; Cook, 1976; Stevens and Moulton, 1978; Greenblatt, Diggs and Aikawa, 1983; Takayanagi *et al.*, 1987). There is also evidence of cellular participation *in vivo* when, following infection, the numbers of macrophages increase in mice infected with *T. brucei* (Murray *et al.*, 1974a;), while the metabolic activity of the mononuclear phagocytic system (MPS) also increases coinciding with heightened phagocytic clearance of parasites from blood (Ferrante *et al.*, 1978; Holmes *et al.*, 1979; MacAskill *et al.*, 1980).

During the development of *T. congolense* induced local skin reactions in sheep, numerous trypanosomes and cells including macrophages/dendritic cells migrate from the skin to the lymph node through the afferent lymph seven to nine days p.i. (Section 6.3.1. and 6.3.2.). In comparison, lower numbers of trypanosomes leave by the efferent lymphatics from lymph nodes draining local skin reactions. This suggests that active destruction of parasites is taking place, probably during transportation of trypanosomes into the draining lymph nodes. In the following study, the fate of trypanosomes in the afferent lymph and the involvement of

macrophages/dendritic cells in their destruction was investigated by light and electron microscopy.

9.2 Materials and Methods

9.2.1 Afferent lymph draining local skin reactions in sheep infected with *T. congolense*

Lymph was obtained from the pseudoafferent lymphatic ducts of two sheep as described in Section 3.6. Samples were collected from the sheep for two days before infection and from three days after infection. The preparation of Giemsa stained cytocentrifuge smears of lymph samples is described in Section 3.4. Stained slides were observed by light microscopy (x40 objective, and under oil immersion x50 and x100 objective).

For preparation of peripheral lymph cells for transmission electron microscopy (TEM), fresh afferent lymph (20 ml) was centrifuged at 1000 g for 10 minutes. The pelleted cells were resuspended and fixed for two to three hrs in 20 ml of 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 (Appendix II) (Sabatini *et al.*, 1963). Subsequently, the cells were washed three times in 0.1M sodium cacodylate buffer for 20 minutes each by centrifugation at 1000 g for five minutes and then stored in same buffer at 4°C in the dark until processed. The processing, staining and examination of afferent lymph cells by TEM was similar to the techniques previously described for skin biopsies (Section 3.5).

9.3 Results

9.3.1 Light microscopic observations of association of trypanosomes and cells of afferent lymph

Prior to infection macrophages or 'veiled' cells in afferent lymph were observed as large cells with abundant weakly staining azurophilic cytoplasm and oval shaped, polymorphic nucleus. Many trypanosomes were seen in afferent lymph from four to five days after infection. During the peak of parasitosis (six to eight days) trypanosomes were observed adhering to macrophages/dendritic cells with their anterior ends forming numerous clusters which also contained a few lymphocytes

(Figure 9.1). Under phase contrast microscopy, trypanosomes attached to the cell clusters in fresh lymph were seen to be actively motile. As the number of trypanosomes declined in lymph (from day nine onwards) numerous lysed trypanosomes were evident while most of the macrophages/veiled cells were seen containing increased numbers of large cytoplasmic vacuoles which contained cytoplasmic inclusions suggestive of phagocytic activity (Figure 9.2).

9.3.2 Transmission electron microscopic observations

Transmission electron microscopy was used to observe closely the contents of the cytoplasmic inclusions in macrophages. Macrophages/veiled cells from uninfected sheep, and those collected up to three days after infection contained a few, small cytoplasmic vacuoles (Figure 9.3). However, in cells collected from six days after infection profiles of trypanosomes were occasionally seen within cytoplasmic vacuoles. Lymphophagocytosis was also observed to occur as intact lymphocytes could be seen encircled by macrophage/dendritic cell processes (Figure 9.4). From eight days after infection many of the macrophages had large cytoplasmic vacuoles containing trypanosomes in various stages of degeneration (Figure 9.5). Some of the ingested material was not always associated with large cytoplasmic vacuoles but was surrounded by numerous electron dense phagosomes (Figure 9.5). Phagocytosis of intact trypanosomes was not observed but numerous cytoplasmic extensions could be seen engulfing trypanosome remnants (Figure 9.5). As the number of trypanosomes in the lymph declined, the number of macrophages containing trypanosome remnants also declined.

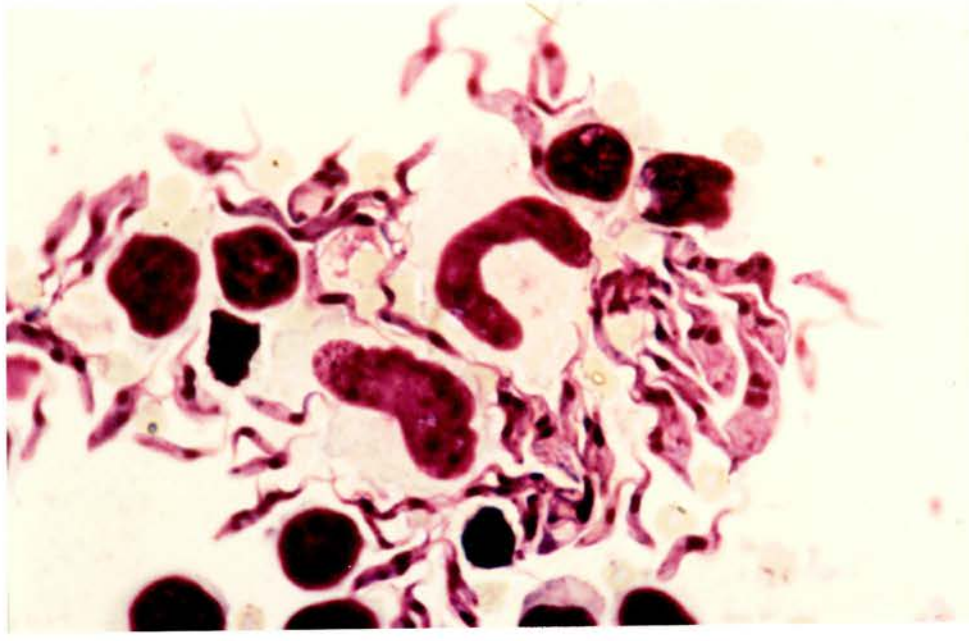
9.4 Discussion

Macrophages are an integral part of the immune response, playing important accessory, effector or regulatory roles (Walker, 1976; Serio, Gandour and Walker, 1979). The involvement of these cells in host protection against facultative and obligate intracellular bacterial invaders *in vivo* is well established (North, 1974). Activated macrophages are important *in vivo* in the destruction of certain viruses and

FIGURE 9.1 Attachment of *Trypanosoma congolense* to macrophages/dendritic cells in the afferent lymph draining from local skin reactions in sheep eight days after infection. Cytocentrifuge smear (Giemsa x1000).

FIGURE 9.2 Peripheral afferent lymph macrophages/dendritic cells 10 days after infection showing presence of numerous cytoplasmic vacuoles apparently containing ingested trypanosome remnants. Fewer trypanosomes are present in the lymph. Cytocentrifuge smear (Giemsa x1000).

9.1



9.2

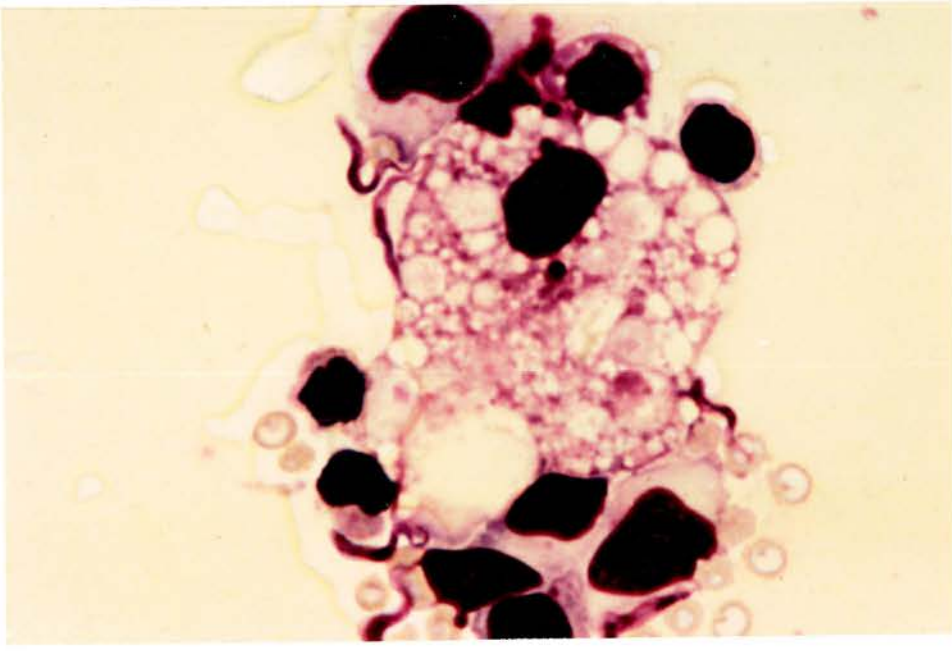
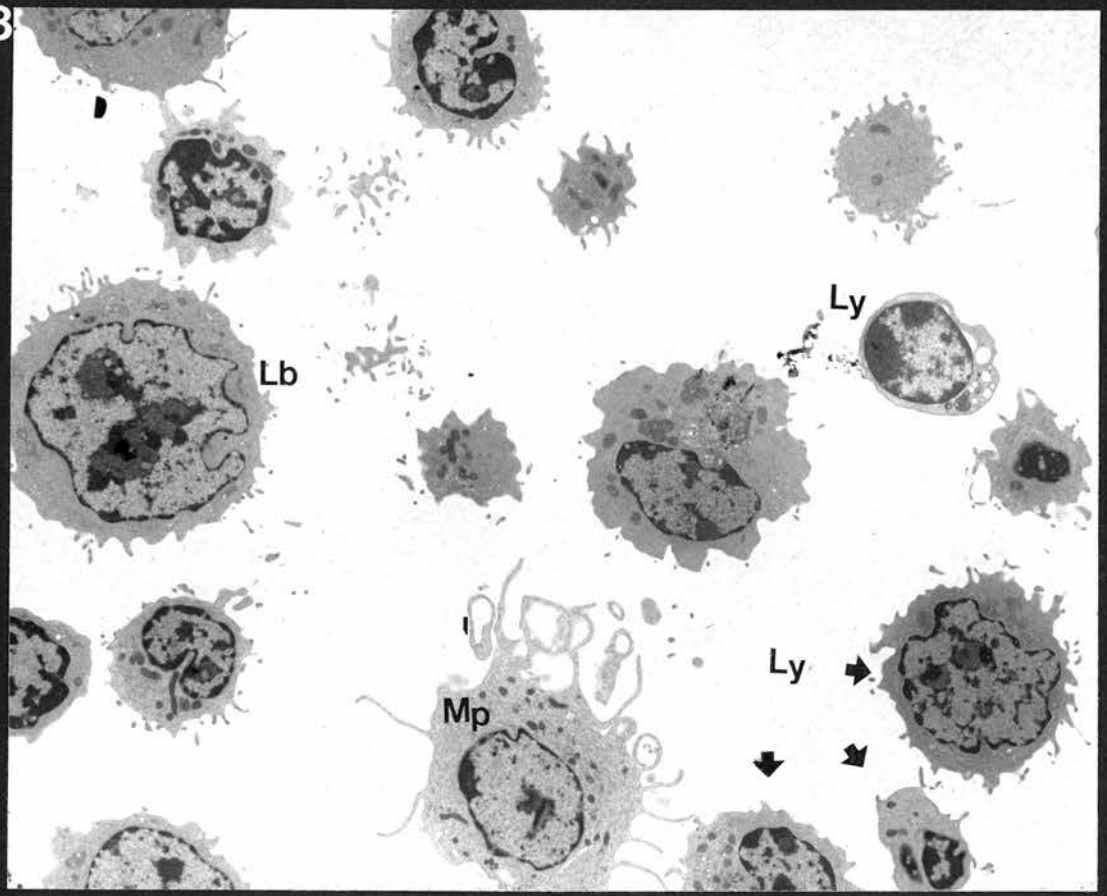


FIGURE 9.3 Low power transmission electron micrograph of cells in a sample of afferent lymph from the skin of uninfected sheep. The lymph contains lymphocytes (Ly), lymphoblasts (Lb) and macrophages (MP) (x2150).

FIGURE 9.4 Transmission electron micrograph of a macrophage (MP) engulfing a small lymphocyte (Ly) in afferent lymph draining local skin reaction in sheep eight days after infection (x4600).

9.3



9.4

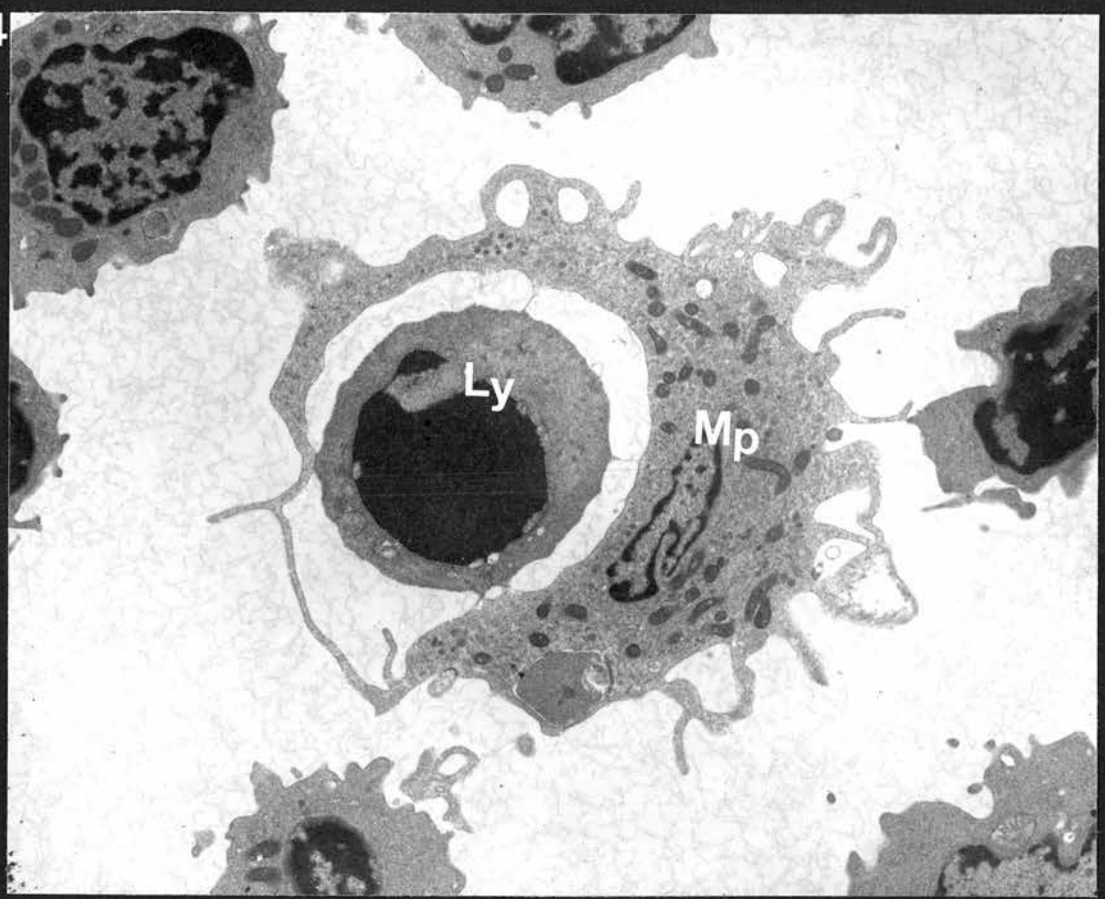


FIGURE 9.5 Transmission electron micrographs of macrophages/dendritic cells in the afferent lymph draining from local skin reactions in sheep eight days after infection with *T. congolense* TREU 1457.

(A) Macrophage apparently in the process of engulfing a degenerating trypanosome (DT). The cell has a long cytoplasmic extension (CE) partially enclosing the trypanosome material. The open arrows indicate the boundary between the macrophage and trypanosome remnants. MN, macrophage nucleus (x7700).

(B) Degenerating trypanosomes (curved arrows) in a host macrophage surrounded by numerous electron dense phagosomes (short solid arrows). Trypanosome remnants are also present extracellularly (curved arrows). f, flagella; MN, macrophage nucleus (X10000).

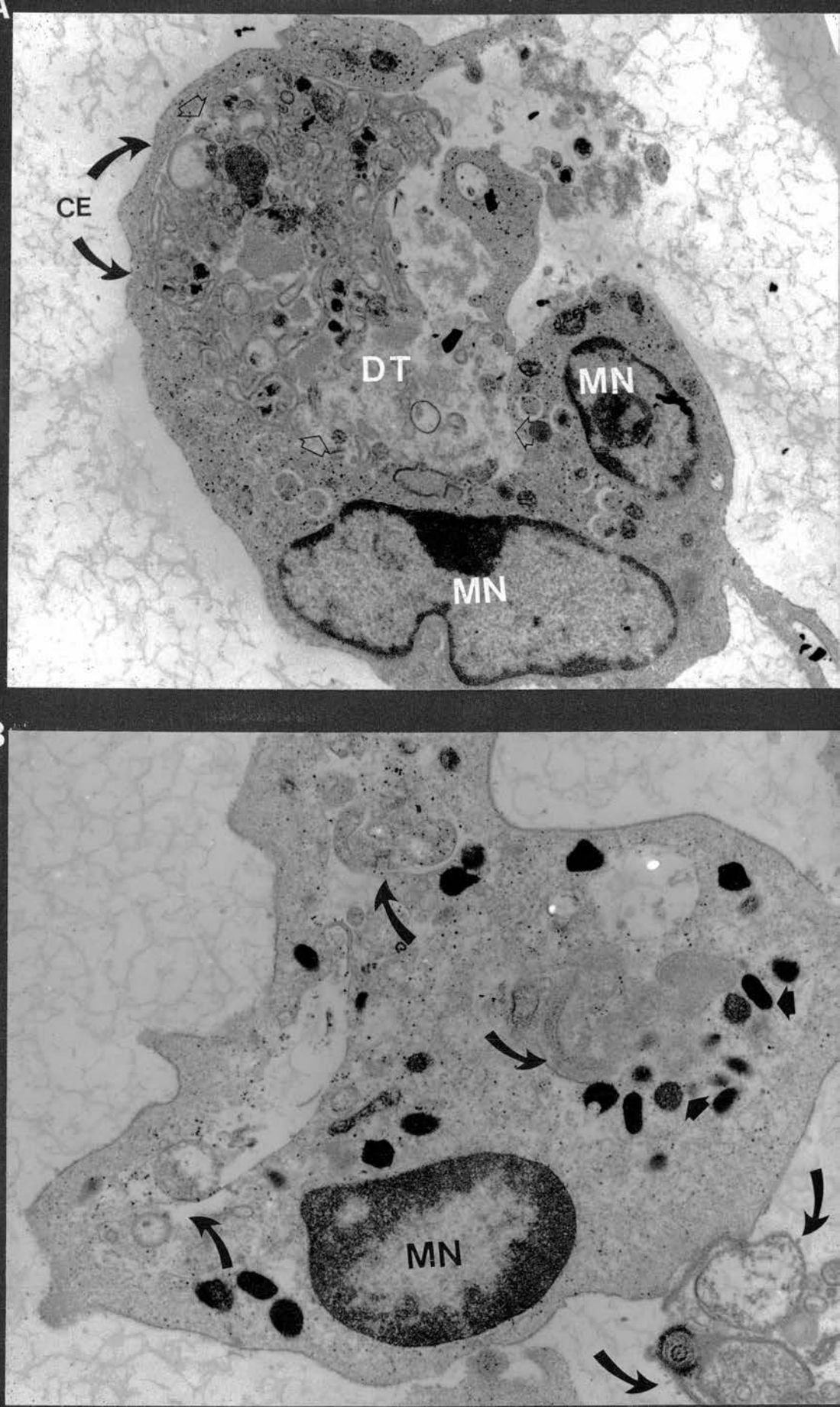
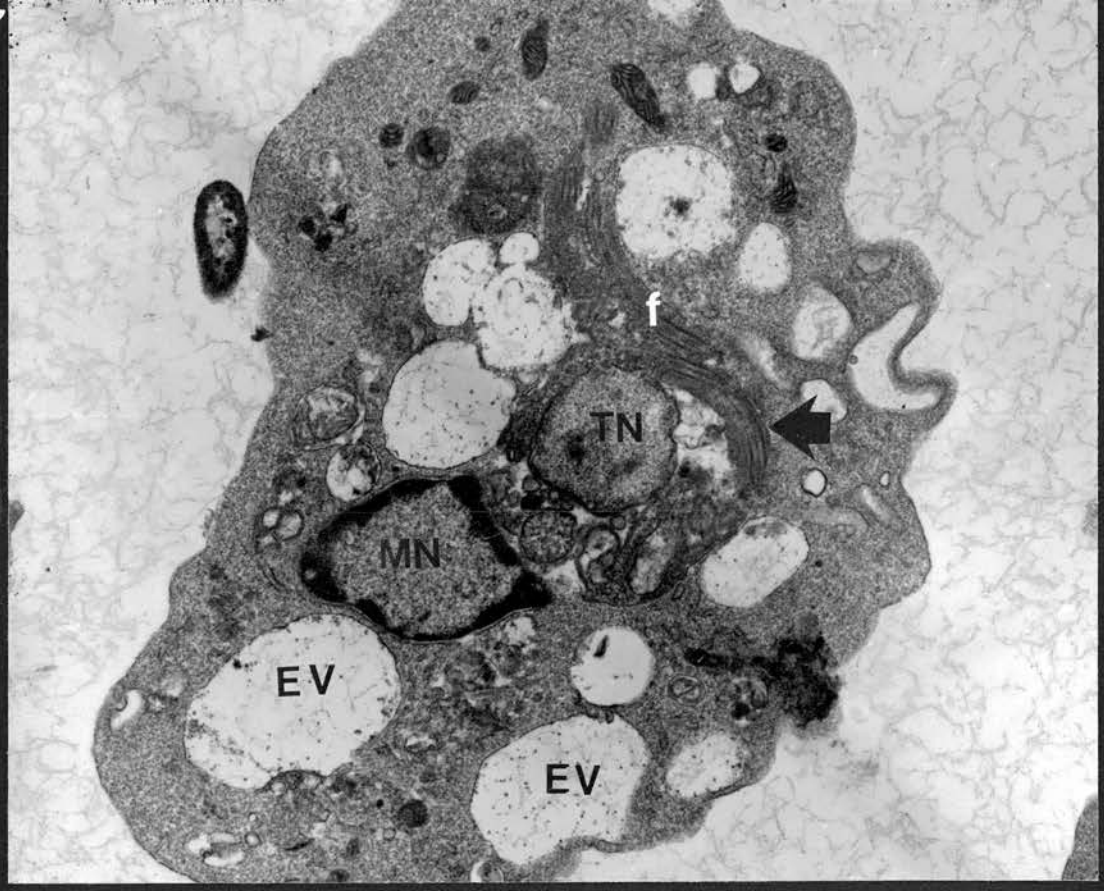
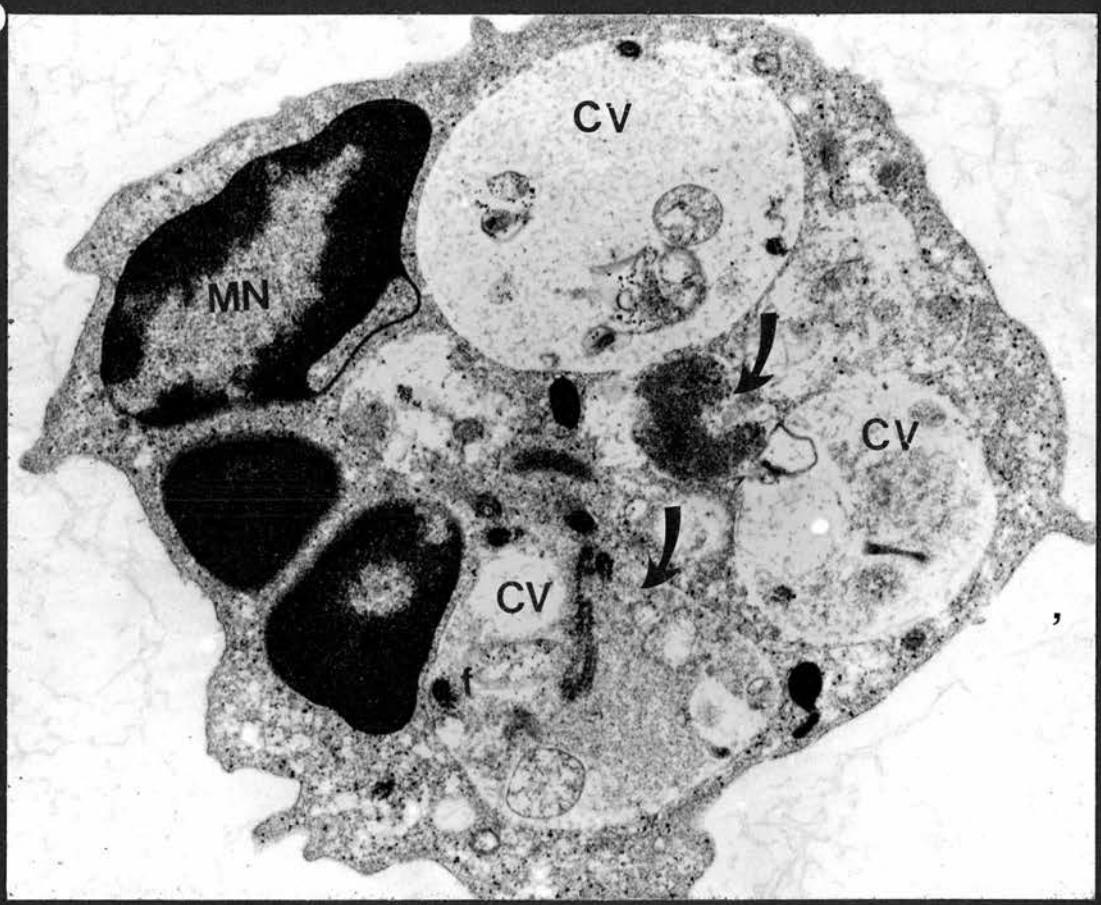

A
CE
DT
MN
MN
B
MN

FIGURE 9.5 Transmission electron micrographs of macrophages/dendritic cells in the afferent lymph draining from local skin reactions in sheep eight days after infection.

(C) A partially degenerated trypanosome (arrow) in a macrophage/dendritic cell. Numerous empty cytoplasmic vacuoles (EV), some containing flagellar sections (f) are present. MN, macrophage nucleus; TN, trypanosome nucleus (x7700).

(D) Cytoplasmic vacuoles (CV) in macrophages/dendritic cell containing cross sections of trypanosome flagellae (f) and other degenerating trypanosome material (arrows). MN, macrophage nucleus (X10000).


C
f
TN
MN
EV
EV


D
CV
MN
CV
CV
f

extracellular parasites (Lefford, 1983; Nelson, 1978). Humoral factors play a major role in immunity to African trypanosomes in vertebrate hosts. However, the mechanisms of parasite killing and elimination probably involves opsonization, phagocytosis and intracellular digestion of trypanosomes by the mononuclear phagocytic system (Lange and Lysenko, 1960; Takayanagi *et al.*, 1974; Takayanagi and Nakatake, 1978; Stevens and Moulton, 1978; Ferrante, 1986). It appears from the evidence presented here that early in the host response the mononuclear phagocytic system is active in the destruction of trypanosomes in the peripheral lymph draining local skin reactions. The initial attachment of *T. congolense* to macrophages/dendritic cells *in vivo* was similar to that described for *T. gambiense* and *T. musculi* when exposed to immune serum in the presence of peritoneal exudate macrophages *in vitro* (Takayanagi *et al.*, 1987; Samarawickrema and Howell, 1988). Phagocytic activity of the cells in the lymph was similar to that shown to occur when *T. lewisi, T. brucei* or *T. gambiense* were incubated *in vitro* with peritoneal macrophages in the presence of specific antibodies (Lumsden and Herbert, 1967; Takayanagi and Nakatake, 1977; Stevens and Moulton, 1978; Greenblatt, Diggs and Aikawa, 1983). The ultrastructure of non-lymphoid mononuclear cells in peripheral lymph accords with previous descriptions (Smith *et al.*, 1970; Hall and Robertson, 1984). The occurrence of several phagocytic cells in the TEM sections indicated that the incidence of phagocytosis probably was high. Phagocytic cells can only be detected in electron microscope studies if the plane of section goes through a phagocytic vesicle which cannot always be the case (Hall and Robertson, 1984).

The actual mechanism which triggers the binding and phagocytosis of trypanosomes in this study has not yet been elucidated. However, many parasites, such as schistosomes, are killed extracellularly after contact with macrophages mediated by specific antibodies or by other undefined factors (Jones and Hancock, 1983). The killing of such extracellular parasites in the presence of antibodies has been likened to an antibody dependent cell-mediated cytotoxicity (ADCC) reaction

against tumour cells (Capron *et al.*, 1982). Secondly, parasites and other antigens opsonized with antibody and/or complement fragments are endocytosed by macrophages via Fc, complement 3, or mannose receptors (Unanue and Allen 1987; Harkiss *et al.*, 1990). The binding and phagocytosis of African trypanosomes by mouse and rat peritoneal macrophages *in vitro* is probably mediated by Fc receptors after lysis of the parasite through antibody-complement reactions or cell-mediated cytotoxicity (Flemmings and Diggs, 1978; Greenblatt *et al.*, 1983). It is possible that specific antibodies were present in the lymph at the time attachment and phagocytosis of trypanosomes was observed (from seven days). Although many intact trypanosomes were observed in the lymph, but not in macrophages under TEM, it is probable that the parasites were first lysed extracellularly prior to ingestion by the phagocytes: the presence of lysed parasites nine days after infection supports this hypothesis. Another trigger for phagocytosis is the non-specific activation of macrophages which occurs during trypanosome infections (Clayton *et al.*, 1980; Grosskinsky *et al.*, 1983). The phagocytosis of intact lymphocytes by macrophages in the afferent lymph might be an indication of such non-specific macrophage activation. The lymphatic system is a major route of migration of trypanosomes from the local skin reaction to the systemic circulation. Although antibody mediated phagocytosis is one way of eliminating trypanosomes *in vivo*, it is not fully effective in preventing infection in susceptible hosts due to antigenic variation. The macrophage phagocytic activity might have immunopathological consequences as well as an immunologically protective function in the infected host. During the early stages of an infection, lymphocytes are triggered by macrophages and antigen leading to production of antibodies and induction of inflammatory reactions that characterize cellular immunity (Unanue and Allen, 1987). Macrophages containing trypanosomes in the lymph might fulfill their antigen presenting functions in the draining lymph node. Infection with African trypanosomes lead to a series of changes in the immune system of the host which results in profound depression of immunological responsiveness to both

trypanosomal and non-trypanosomal antigen (Jayawardena and Waksman, 1977; Hudson and Terry, 1979). Although T and B lymphocytes are directly involved in this process of immune dysfunction it seems that macrophages might also play a key role. In mice infected with *T. brucei* macrophages are capable of mediating immunosuppression *in vivo* after taking up opsonized parasites (Grosskinsky and Askonas, 1981) and elaborating inhibitory factors. Such factors include prostaglandin E_2 (PGE_2) which is capable of inhibiting interleukin-2 (IL-2) and IL-2 receptor expression on T cells (Sileghem *et al.*, 1989a,b). Macrophages from mice infected with *T. brucei* also show reduced ability to present antigens to antigen primed T cells (Bagasra, Schell and Le Frock, 1981). Due to the state of activation of macrophages, they might contribute effectively in the development of anaemia and leucopaenia by taking up and destroying red blood cells coated with antibody-antigen complexes (Fierer and Askonas, 1982) or leucocytes as observed in the present work. Activated macrophages secrete monokines which might be responsible for some of the pathogenic lesions observed in infected animals. Tumour necrosis factor (TNF) or cachectin is one such factor which could be secreted during infection. TNF may be an important mediator of tissue destruction in non antibody-mediated cytotoxicity (Beutler *et al.*, 1985; Beutler and Cerami, 1986). Since dendritic cell/macrophages are not only confined to lymph from skin but are abundant in peripheral lymph from other tissues (Smith *et al.*, 1970; Hall, Hopkins and Orlans, 1977; Hall, 1979) it is possible that these immunopathological effects could be extensive and systemic in trypanosome infected hosts.

CHAPTER TEN
GENERAL DISCUSSION

The need to control effectively animal trypanosomiasis is one of the major priorities in efforts to improve livestock production in Africa. Attempts to reduce the disease prevalence by vector control and use of trypanocidal drugs have been expensive and relatively unsuccessful. Vast areas are still infested with tsetse while areas cleared of tsetse are prone to reinfestation (MacLennan, 1981). At the same time, the numbers of trypanocidal drugs which are effective against trypanosomes are declining due to the emergence of drug resistance, resulting in a breakdown of chemotherapeutic and chemoprophylaxis control regimens (Küpper and Wolters, 1983; Pinder and Authie, 1984). Since it is unlikely that a suitable vaccine against tsetse-transmitted trypanosomiasis will be developed, the use of trypanocidal drugs will need to be assessed more carefully than has been done previously. Already new information is becoming available on the chemoprophylactic efficiency of Samorin in relation to challenge with metacyclic *T. congolense* and *T. vivax*. Acquisition of resistance by cattle maintained in tsetse-infested areas under chemotherapy also provides a means by which effective control can be applied (Wilson *et al.*, 1975, 1976; Murray and Urquhart, 1977). However, there is evidence that the development of immunity following treatment with the trypanocidal drugs Berenil and Homidium depends on both the timing of treatment and the stage of infection (Luckins *et al.*, 1983; Dwinger *et al.*, 1990). In cyclically transmitted infections, the initial development and proliferation of trypanosomes in the skin is of prime importance in determining the establishment of infection and outcome of the disease. Homologous immunity is thought to be directed against metacyclic trypanosomes in the skin, while trypanotolerance also appears to be associated with non-immune control of trypanosome replication in the skin (Murray *et al.*, 1982; Akol *et al.*, 1986a). It is therefore essential to obtain a greater understanding of this early host-trypanosome interaction in order to define the mechanisms of generation or breakdown of immunity.

Tsetse flies introduce only low numbers of metacyclic trypanosomes but they undergo extensive multiplication so that by seven to 10 days after initial infection, large numbers of parasites are present in the skin (Gray and Luckins, 1980; Emery and Moloo, 1980, 1981; Akol and Murray, 1982; Dwinger *et al.*, 1987). Although it is not possible to quantitate the numbers of parasites present in the skin, their increase can be judged from the number of trypanosomes in the draining afferent lymph. Following an initial intradermal inoculation of 4×10^5 metacyclic trypanosomes, few parasites were present in afferent lymph for the first six days (Chapter 6). By seven to 10 days p.i., up to 2×10^9 trypanosomes were collected from afferent lymph in one day and parasitosis persisted at the level of more than 1×10^5 per day 21 days after infection. The delay in appearance of trypanosomes in afferent lymph suggests that parasites initially reside in the skin where they possibly transform to a dividing intracutaneous stage (Dwinger *et al.*, 1987). After this transformation the trypanosomes no longer possess the ability to remain in the dermal tissues and begin to leave in large numbers. Their presence in the afferent lymph indicates the importance of the draining lymphatics in the systemic dissemination of the trypanosomes. This route of migration continues via the lymph nodes, where marginal sinuses contain many trypanosomes, and thence to the efferent lymphatics.

The VAT repertoire of metacyclic trypanosomes of *T. congolense* is limited (Crowe *et al.*, 1983) and therefore it is an advantage for the parasite to generate new VATs for effective establishment of infection. This appears to take place in the skin as the trypanosomes proliferate. Although trypanosomes in the skin continue to express a range of metacyclic VATs, up to 50% express non-M-VATs seven to 10 days after infection (Luckins *et al.*, 1990). Similarly, only a proportion of trypanosomes in afferent lymph continue to express M-VATs up to 10 days after infection and trypanosomes in efferent lymph do not appear to express any M-VATs as early as seven days post-infection (Sutherland, personal communication). Progressive increase in expression of non-metacyclic VATs therefore occur during

development and migration of trypanosomes from the skin through the lymphatic system. The presence of many lysed trypanosomes in the skin, afferent and efferent lymph (Chapters 4, 6 and 7) and evidence of active phagocytosis by macrophages/dendritic cells in afferent lymph may be evidence of accompanying destruction of those trypanosomes expressing M-VATs. The presence in efferent lymph of lower numbers of trypanosomes than in afferent lymph, the persistence of parasitosis and the non-expression of M-VATs on these parasites indicate that the draining regional node as well as the skin is a site of destruction of earlier infecting M-VATs and generation of trypanosomes expressing new VATs. The destruction of trypanosomes in these compartments might be mediated by specific anti-metacyclic VAT antibodies. Anti-M-VAT antibodies have been demonstrated in the efferent lymph of cattle, sheep and goats 14 days after *T. congolense* infection (Akol and Murray, 1986; Dwinger *et al.*, 1990; Luckins *et al.*, 1990). The presence of plasma cells and B cell aggregates in the skin five to seven days after infection (Chapter 4) suggests that local antibody production might also be taking place. Preliminary studies (not described), although not conclusive, indicate that anti-metacyclic antibodies are also present in afferent lymph. The technique of cannulation of afferent and efferent lymphatics draining local skin reactions therefore has great potential as a means of studying trypanosome biology and biochemistry, in particular antigenic variation, during transformation from metacyclic to mammalian forms as well as the initial host immune responses to trypanosomes.

The skin appears to provide a suitable medium for trypanosome proliferation and expansion of M-VATs which is an important aspect of induction of protective immunity in rabbits and cattle after trypanocidal drug therapy (Luckins *et al.*, 1983; Akol *et al.*, 1986a; Taiwo *et al.*, 1990). This is however, related to the timing of drug therapy, stage of development of local skin reactions and persistence of large numbers of trypanosomes within the skin. Rabbits became immune to homologous challenge if they were treated seven days after initial *T. congolense* infection (Luckins *et al.*,

1983) while cattle do not appear to develop comprehensive immunity if treated less than 12 days after original infection (Akol *et al.*, 1986a). This might be due to differences in the course of development of local skin reactions and the rate of generation of new VATs in the two animal species. Homologous immunity in sheep appeared to be effected in the skin, as no local reactions were detected at sites of homologous challenge of immune sheep (Chapter 4). This is supported by the absence of trypanosomes in afferent lymph up to 16 days after homologous challenge of immune sheep (Chapter 6) and in efferent lymph draining sites of homologous challenge in immunized cattle and goats (Akol and Murray, 1986; Dwinger *et al.*, 1990). Presence of specific antibodies in the skin probably ensures rapid killing of trypanosomes and therefore the absence of local skin reactions.

Goats cyclically infected with *T. vivax* fail to develop comprehensive homologous immunity after chemotherapy (Vos *et al.*, 1988b). This seems to be linked to development of only transient, small local skin reactions compared to either *T. brucei* or *T. congolense* (Emery and Moloo, 1980, 1981; Dwinger *et al.*, 1988). It has been suggested that *T. vivax* rapidly leaves the skin after inoculation (Dwinger *et al.*, 1988; Vos *et al.*, 1988b) and does not proliferate in high numbers. It is therefore possible that the rate of generation of new VATs and exposure to the local host immune sytem is limited and insufficient to induce consistent protective immunity.

Interference in establishment of secondary trypanosome infections by existing ones appears to be mediated in the skin or local draining lymph nodes. No skin reactions developed in infected sheep at site of superinfection with large numbers of *T. congolense* of an heterologous serodeme (Chapter 4) and trypanosomes were absent in efferent lymph from lymph nodes draining similar sites up to eight days after challenge (Chapter 7). This may be a reflection of the presence in the skin and lymph node of factors other than antibodies which inhibit the replication and establishment of superinfecting trypanosomes. Interference appears to develop approximately 14 days after infection (Luckins and Gray, 1983; Dwinger *et al.*, 1989). Since this

phenomenon is absent after trypanocidal therapy, it is apparent that the inhibitory factors are not anti-trypanosome antibodies and that they are produced or induced by active trypanosome infections only. Identification of such factors either in draining lymph or blood might be of importance in the development of any future biologically based trypanosomiasis control strategies.

Trypanosome proliferation in the skin and subsequent migration into the systemic circulation through the lymphatic system elicited marked cellular responses in the skin, draining lymph nodes as well as in afferent and efferent lymph. These cellular responses and specifically the changes in proportions and numbers of various leucocyte phenotypes indicate a sequence of inflammatory, immunological and pathological processes which, although not able to prevent the establishment of infection successfully obviate reinfection with an identical trypanosome serodeme. The first four to five days after infection is only characterized by presence of trypanosomes in the dermal collagen (Chapter 4), cytological changes of mast cells but a lack of any other cellular responses either in the skin, draining lymphatics, lymph nodes or peripheral blood. This probably refects the latent phase in which trypanosomes proliferate, invade the dermis in large numbers and thus elicit inflammatory responses after five days of infection. The initial inflammatory responses in the various compartments (five to seven days) coincide with appearance of large numbers of trypanosomes. In the skin, this response is characterized histologically by infiltration of mononuclear cells and neutrophils into the inoculation site (Chapter 4, Emery and Moloo, 1980; Akol and Murray, 1982; Dwinger *et al.*, 1987). Neutrophils appear to be confined to the skin reaction as very few of these cells are present in afferent lymph (Chapter 6) and none are present in draining lymph nodes or efferent lymph (Chapter 5 and 7). This is in contrast to inflammatory responses in the skin induced by chemical agents such as DNFB which are characterized by an influx of neutrophils in the skin and afferent and efferent lymph (Hall and Smith, 1971). Since neutrophils are not present in large numbers in the skin

after seven days of infection, it is possible that after initial recruitment by chemotactic factors, they fulfill their phagocytic function and are then destroyed within the skin.

During the same period (five to seven days) the mononuclear cell infiltrate in the skin contains numerous B cells many in focal aggregates (Chapter 4). These cells appear to proliferate and do not leave the skin in large numbers since very few B cells are present in afferent lymph despite the increased cellular response (Chapter 6). This might indicate an impedence of B cell passage into afferent lymph during the early phase of infection. However, during the same period, marked B cell responses are observed in draining lymph nodes and efferent lymph but not in peripheral blood. This finding indicates that the B cell response in the lymph node and efferent lymph is not due to increased migration from the skin but to local proliferation within the node probably caused by influx of large number of trypanosomes, trypanosome antigens or antigen specific T cells and macrophage/dendritic cells. Trypanosomes contain B cell mitogenic factors (Tizard *et al.*, 1978) which might elicit preferential B cell stimulation. Some of the T cells migrating from the skin into the draining lymph node might be antigen specific but this remains to be investigated. Afferent macrophage/dendritic cells containing phagocytosed trypanosomes entering the lymph node might also lead to marked B cell proliferation. In the mouse, macrophages activated following ingestion of opsonized trypanosomes have been shown to present antigen to T cells which in turn secrete B cell mitogenic factors *in vitro* (Grosskinsky and Askonas, 1981; Sacks *et al.*, 1982; Grosskinsky *et al.*, 1983; Bancroft and Askonas, 1985). The timing of this response indicates that it is unlikely that B cells in the local skin reaction recirculate from the draining lymph nodes.

During the same period T lymphocytes are present in the skin in similar numbers to B cells but are more diffusely distributed. More $CD4^+$ cells than $CD8^+$ cells are present but very low numbers of SBU-$T19^+$ cells (Chapter 4). This is reflected by changes in afferent lymph where the cellular response is predominantly due to an increase in $CD4^+$ cells and to a lesser extent $CD8^+$ cells. At this time there

is an increase in the numbers of $CD1^+$ cells and macrophage/dendritic cells in afferent lymph. The proportions of T cell subpopulations in efferent lymph decrease but their absolute numbers increase. The decrease in T cell proportions is due to the more marked increase in B cells.

The second phase of response of the local skin reaction (after 10 days) is characterized by the presence of few B cells. The cells appear to leave the skin at this time since their numbers and proportions increase in afferent lymph. These B cells can only be coming from the skin and not recirculating as most of the cells were removed by the indwelling lymphatic cannula. The lymph node B cell response continues throughout the course of the local skin reaction. A marked B cell response was observed in peripheral blood after regression of the local skin reaction (Chapter 8). This might be a reflection of B cell lymphoproliferative changes in draining lymph nodes, efferent lymph and probably other systemic lymphoid tissues following onset of parasitaemia. In this way, antigen specific effector and memory B cells would be disseminated systemically. This might explain the lack of development of homologous immunity of infected animals if they are treated too early, prior to the full development and resolution of local skin reactions (Akol and Murray, 1983).

During this second phase of cellular response, T cells especially $CD8^+$ cells persist in the skin. The afferent lymph still contains more $CD4^+$ cells than $CD8^+$ indicating an increased migration of $CD4^+$ from the skin compared to $CD8^+$ cells. At the same time, many $CD8^+$ cells are present in the medulla of regional lymph nodes. This disproportionate increase in $CD8^+$ cells compared to $CD4^+$ cells might indicate an ineffective immune response (Modlin *et al.*, 1983). Alternatively, $CD8^+$ cells which are suppressor/cytotoxic T cells might be responsible for the non-specific immunosuppression observed in trypanosomiasis.

The cellular changes associated with homologous challenge of immune sheep are minimal. A lack of development of local skin reactions at the inoculation site and absence of trypanosomes or cellular responses in afferent lymph indicates that

immunity might be mediated by humoral antibodies present in the skin which rapidly kill trypanosomes. It is possible that induction of immunity is dependent on development of local skin reactions and probably T cell help. However, more work is required to compare immune animals and those undergoing primary infections. Interference phenomenon also appears to be mediated by humoral factors rather than by cells themselves. This is indicated by lack of local skin reactions and absence in efferent lymph of trypanosomes for up to nine days post-challenge. Equally, factors probably produced or induced by living trypanosomes might be responsible for this delay in establishing infection. However, further work to identify such factors should be pursued since it might have promise in future control of the disease.

The above responses in the local skin reactions, draining lymph and lymph nodes are ineffective in preventing the establishment of *T. congolense* infection in sheep since, by this time, new trypanosome VATs have already emerged. However, the responses appear to be essential in the subsequent development of protective immunity against homologous challenge if the infected animals are treated with trypanocidal drugs (Taiwo *et al.*, 1990). It would be useful to compare the cellular responses of animals with differing susceptibilities to trypanosome infections as a superior and early response in the skin and draining lymph node might be responsible for the trypanotolerance exhibited by some domestic and wild animals (Akol *et al.*, 1983).

More work, to answer some of the questions raised in this study, needs to be pursued in order to elucidate further the events which occur during the initial host-trypanosome interaction in the skin and draining lymph nodes. The cellular phenotypes in trypanotolerant animals should be compared to those in susceptible animals undergoing primary infections with a view to investigating any cellular basis of superior resistance. In addition, the pseudoafferent and efferent lymph from the local skin reactions could be assayed for presence of specific anti-trypanosome (M-VAT) antibodies, antigen specific cells, or monokines (such as interleukins,

IFN-y or TNF) generated by cells infiltrating the skin and which might be responsible for B cell response in draining lymph nodes. Other humoral factors which mediate interference or trypanoresistance can also be investigated. The pseudoafferent lymph is also a source of numerous trypanosomes migrating from the local skin reaction which could be useful in studies on transformation from metacyclic to bloodstream forms *in vivo* and also for studies or antigenic variation.

In conclusion, this study provides a useful model for future studies on immunity and trypanoresistance in domestic animals. The key to understanding these processes and the pathogenesis of lesions in various organs in trypanosome infected hosts might lie in the determination of mechanisms which induce development of local skin reactions.

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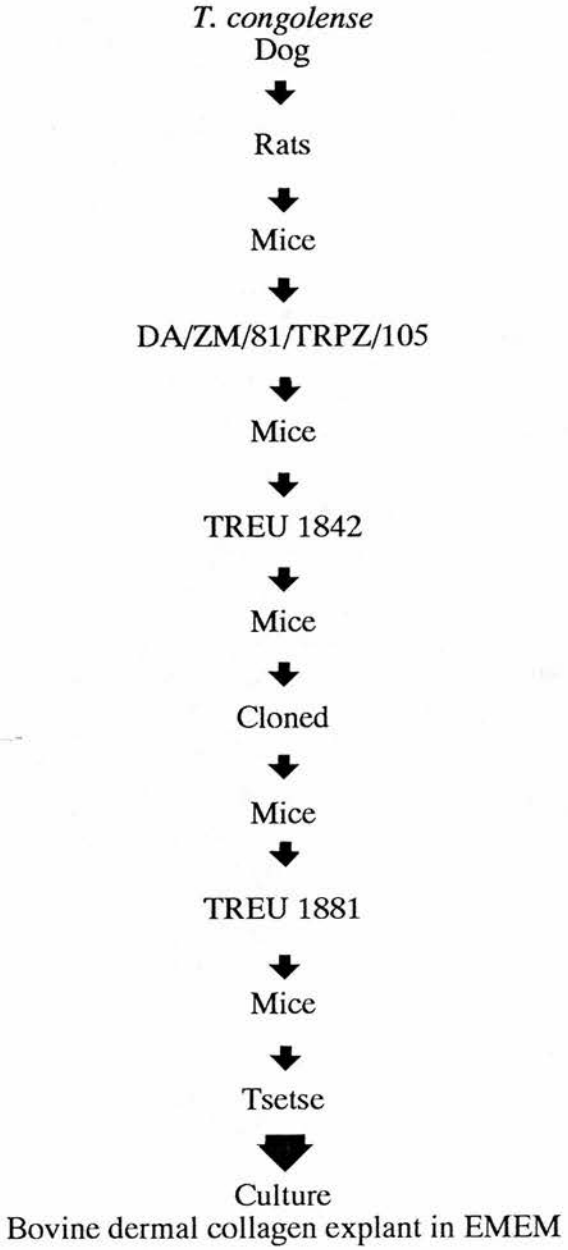
APPENDICES

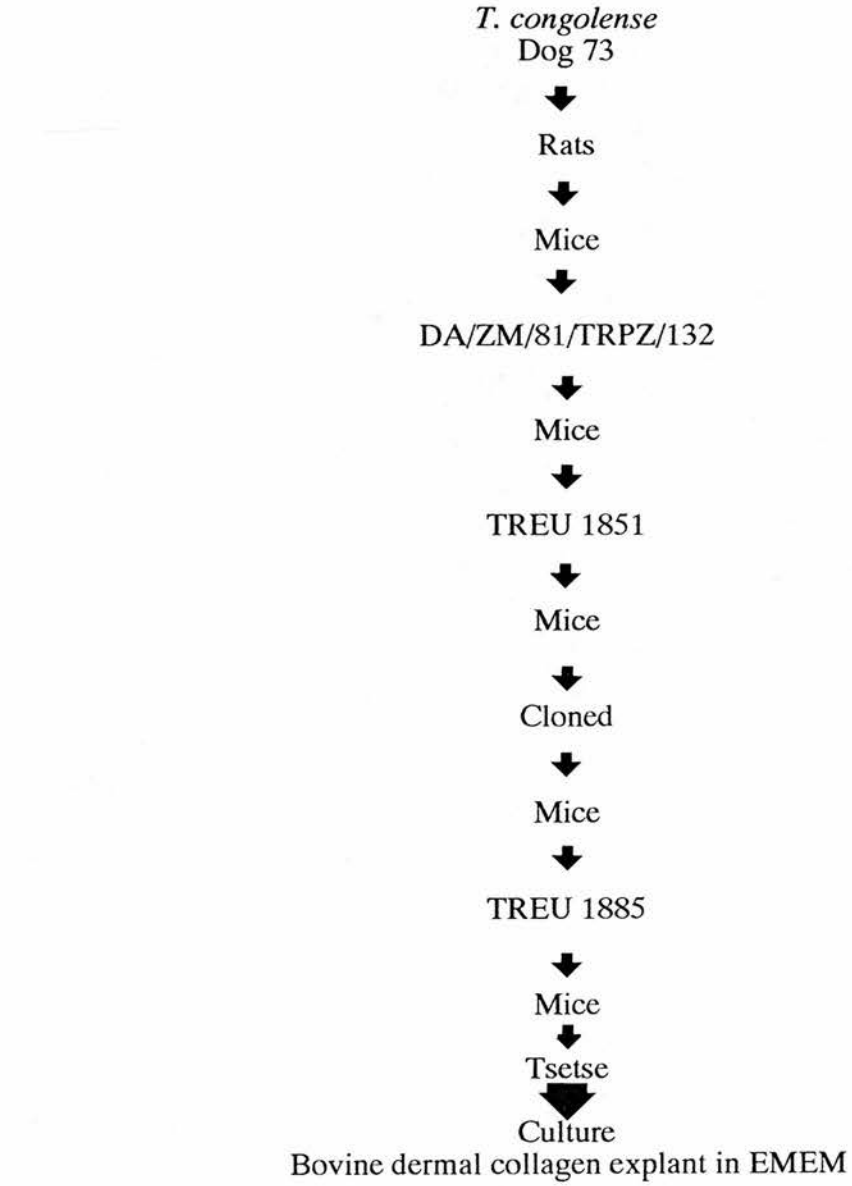
APPENDIX I
DERIVATION OF TRYPANOSOMES

T. congolense
Cow
↓
Mouse
↓
ZARIA/67/LUMP/79
↓
TREU 1172
↓
Mouse
↓
TREU 1290
↓
Mouse
↓
Cloned
↓
Mouse
↓
TREU 1457
↓
Mice
↓
Tsetse
↓
Culture
Bovine dermal collagen explant in EMEM

Zaria, Nigeria 1967



Kakumbi, Chipata District, Zambia, 1971



Kakumbi, Chipata District, Zambia, 1981

APPENDIX II
BUFFER SOLUTIONS AND REAGENTS

1. **CACODYLATE BUFFER (0.1M, pH 7.4)**
   - Solution A. 0.2M sodium cacodylate ($Na(CH_3)_2$ As $0_2.3H_20$), 42.8 g in 100 cm^3 of distilled water
   - Solution B. 0.1N Hydrochloric acid (HCL)
   - Solution C. 0.5M Calcium Chloride ($CaCl_2$, anhydrous)

   25 cm^3 of Solution A and 1.35 cm^3 of Solution B mixed, adjusted to pH 7.4 and made up to 50 mls with distilled water.

2. **DIETHYLAMINOETHYL CELLULOSE (DEAE-52)** for anion exchange Preparation:
   a) DEAE-52 suspended in PSG in a ratio of 50 g/200 ml.
   b) pH adjusted to 8.0 with Molar orthophosphoric acid added dropwise.
   c) The suspension is washed four times with a volume of PSG equal to that which DE-52 was originally suspended in.
   d) Final slurry stored at 4°C until used.

3. **4% GLUTARALDEHYDE FIXATIVE**

25% aqueous glutarldehyde	8.0 cm^3
0.1M cacodylate buffer	42.0 cm^3

4. **IMMUNOFLUORESCENCE BUFFER (IMFB)**

Bovine serum albumin (Sigma)	1%
Sodium azide	0.1%
(Heparin	20 I.U./ml)

Made up in PBS pH 7.2

5. **IMMUNOPEROXIDASE BUFFER (IMPB)**

Bovine serum albumin (Sigma)	0.1%
Normal sheep serum	2%
Polyoxyethyl sorbitan mono-oleate (Tween 20)	0.08%

Made up in PBS pH 7.2

6. **IMMUNOPEROXIDASE SUBSTRATE**
   a) 10 mg diaminobenzidine tetrahydrochloride (DAB)* dissolved in 20 mls of PBS (pH 7.2) and filtered to remove insoluble particles.
   b) 40 ml of Hydrogen peroxide (30% H_20_2) added to DAB solution just before use.

* Listed as a potential carcinogen. To be handled with care.

7. **PHOSPHATE BUFFERED SALINE** (PBS, pH 7.2)

Sodium chloride (NaCl)	8.00 g $litre^{-1}$
Potassium chloride (KCl)	0.20 g $litre^{-1}$
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g $litre^{-1}$

Potassium dihydrogen phosphate
(KH_2PO_4) 0.20 g litre^{-1}
Dissolved in 1000 ml of distilled water

8. **PHOSPHATE BUFFERED SALINE GLUCOSE** (PSG, pH 8.0)
Preparation of stock phosphate saline solution (PS stock)

Disodium hydrogen phosphate, anhydrous (Na_2HPO_4)	13.48 gl^{-1}
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_20$)	0.78 gl^{-1}
Sodium chloride (NaCl)	4.25 gl^{-1}

Made up in warm distilled water

To prepare PSG for use, PS stock diluted 6:4 in distilled water and 1% glucose (10 gl^{-1}) added.

9. **TRIS-AMMONIUM CHLORIDE**
Materials
0.17M Tris (hydroxymethyl) aminomethane (20.6 gl^{-1})
0.16M Ammonium chloride (8.30 gl^{-1})

Method
Ten mls of 0.17M Tris added to 90 mls of 0.16M ammonium chloride and adjusted to pH 7.2 using 1M HCL.

APPENDIX III
PERIPHERAL LYMPH CANNULATION DATA

TABLE A 3.1 Lymph flow, cellular responses and parasite kinetics in peripheral afferent lymph of sheep 016 after infection in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^6$)	Parasitosis (log_{10} tryps/ml)
1	60 (18 hrs)	3.6	10.8	3.9
2	110	2.3	10.5	3.9
3	160	2.1	14.0	-
4	170	2.0	14.1	-
5	210	2.2	19.3	4.5
6	235	1.9	18.6	6.6
7	182	2.0	15.2	6.9
8	135	3.2	18.0	7.2
9	150	7.5	46.9	7.2
10	164	14.2	97.0	6.3
11	166	14.6	101.0	4.8
12	166	14.6	101.0	4.8
13	110	18.4	84.3	6.3
14	122	14.2	72.2	4.8
15	130	12.9	69.9	6.6
16	123	13.3	68.1	6.3
17	156	14.6	94.9	5.4
18	140	15.0	87.5	4.8
19	153	14.3	91.2	6.0
20	150	14.3	89.4	5.4
21	110	10.3	47.2	5.7
22	68	15.0	42.5	5.4

TABLE A 3.2 Differential leucocyte counts of peripheral afferent lymph cells of sheep 016 infected with *T. congolense* TREU 1457. Absolute values (x10^6/hr) in brackets

Days after infection	Small lymphocytes(%)	Large lymphocytes(%)	Macrophages/ dendritic cells(%)	Granulocytes (%)
1	41.0 (4.4)	0.0 (0)	5.5 (0.6)	53.5 (5.8)
2	79.0 (8.3)	0.0 (0)	8.5 (0.9)	12.5 (1.3)
3	86.0 (12.0)	0.0 (0)	10.0 (1.4)	4.0 (0.6)
4	93.0 (13.1)	0.0 (0)	3.0 (0.4)	4.0 (0.6)
5	87.5 (16.9)	0.5 (0.1)	8.5 (1.6)	3.5 (0.7)
6	90.0 (16.8)	0.0 (0)	6.0 (1.1)	4.0 (0.7)
7	87.0 (13.2)	0.0 (0)	8.0 (1.2)	5.0 (0.8)
8	86.0 (15.5)	2.5 (0.5)	9.5 (1.7)	2.0 (0.3)
9	88.5 (41.5)	3.5 (1.6)	8.0 (3.8)	0.0 (0)
10	89.5 (86.8)	6.0 (5.8)	4.0 (3.9)	0.5 (0.5)
11	89.5 (90.4)	3.5 (3.5)	7.0 (7.1)	0.0 (0)
12	86.5 (87.4)	3.5 (3.5)	9.0 (9.1)	1.0 (1)
13	92.0 (77.6)	5.0 (4.2)	3.0 (2.5)	0.0 (0)
14	84.0 (60.6)	7.0 (5.1)	9.0 (6.5)	0.0 (0)
15	93.5 (65.4)	2.5 (1.7)	4.0 (2.8)	0 (0)
16	89.0 (60.6)	8.0 (5.4)	3.0 (2.1)	0 (0)
17	88.5 (84.0)	6.5 (6.2)	5.0 (4.7)	0 (0)
18	84.0 (73.5)	12.5 (10.9)	3.5 (3.1)	0 (0)
19	85.0 (77.5)	12.0 (10.9)	3.0 (2.8)	0 (0)
20	81.5 (72.9)	15.0 (13.4)	3.0 (2.7)	0.5 (0.4)
21	83.0 (39.2)	13.5 (6.4)	3.5 (1.6)	0 (0)
22	79.4 (33.7)	17.8 (7.6)	2.8 (1.2)	0 (0)

TABLE A 3.3 T lymphocyte subpopulation dynamics in peripheral afferent lymph of sheep 016 infected with *T. congolense* TREU 1457. Absolute cell output x 10^6/hr (relative proportions as percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
1	4.23 (81.3)	2.74 (52.7)	0.57 (10.9)	0.55 (10.9)
2	8.10 (88.1)	5.13 (55.8)	1.47 (16.0)	1.10 (12.0)
3	11.77 (87.8)	7.97 (59.5)	2.63 (19.6)	1.49 (11.1)
4	10.84 (80.3)	4.93 (36.5)	4.41 (32.7)	1.67 (12.4)
5	16.40 (85.0)	6.97 (38.7)	2.83 (15.3)	2.39 (12.9)
6	16.09 (89.9)	10.20 (57.0)	2.90 (16.2)	1.45 (8.1)
7	13.28 (92.2)	8.48 (58.9)	3.24 (22.5)	1.38 (9.6)
8	15.55 (90.4)	10.37 (60.3)	3.23 (18.8)	0.88 (5.1)
9	44.27 (94.4)	32.69 (69.7)	10.69 (22.8)	2.25 (4.8)
10	90.99 (93.8)	78.98 (81.1)	22.70 (23.4)	3.78 (3.9)
11	95.04 (94.1)	68.98 (68.3)	25.55 (25.3)	7.37 (7.3)
12	94.03 (93.1)	63.73 (63.1)	26.77 (26.5)	4.85 (4.8)
13	75.20 (89.2)	52.86 (62.7)	22.68 (26.9)	4.47 (5.3)
14	54.51 (75.5)	42.09 (58.3)	12.60 (17.4)	3.47 (4.8)
15	ND ND	ND ND	ND ND	ND ND
16	59.11 (76.8)	42.83 (62.9)	15.91 (23.4)	4.63 (6.8)
17	73.64 (77.6)	60.26 (63.5)	14.99 (15.8)	4.08 (4.3)
18	66.50 (76.0)	51.45 (58.8)	15.73 (17.9)	4.64 (5.3)
19	68.13 (74.7)	52.44 (57.5)	13.59 (14.9)	4.29 (4.7)
20	64.54 (72.2)	44.16 (49.4)	10.91 (12.2)	3.31 (3.7)

TABLE A 3.4 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$, and SIg$^+$ cells in peripheral afferent lymph of sheep 016 infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	CD1$^+$ cells	MHC Class II$^+$ cells	CD45R$^+$ cells	SIg$^+$ cells
1	1.48 (29.5)	1.80 (35.9)	0.96 (19.1)	1.81 (9.7)
2	0.60 (6.5)	4.03 (43.8)	0.81 (8.8)	2.27 (21.5)
3	1.23 (9.2)	5.63 (42.0)	1.03 (7.7)	2.41 (13.3)
4	1.35 (10.0)	3.63 (26.9)	0.93 (6.9)	2.14 (6.6)
5		4.92 (26.6)	1.78 (9.6)	3.37 (7.9)
6	1.37 (7.7)	7.36 (41.1)	2.86 (16.0)	1.97 (11.0)
7	1.43 (9.9)	7.78 (54.0)	0.91 (6.8)	2.63 (14.4)
8	1.55 (9.0)	8.29 (48.2)	1.12 (6.5)	2.06 (7.3)
9	3.00 (6.4)	20.35 (43.4)	2.35 (5.0)	8.06 (11.2)
10	3.49 (3.6)	35.60 (36.7)	3.88 (4.0)	7.37 (3.6)
11	3.23 (3.2)	34.85 (34.5)	5.05 (5.0)	8.48 (6.4)
12	5.76 (5.7)	21.58 (25.6)	9.49 (9.4)	13.74 (9.8)
13	2.78 (3.3)	18.20 (21.6)	9.61 (11.4)	12.56 (15.0)
14	4.69 (6.5)	20.36 (28.2)	5.70 (7.9)	13.50 (11.5)
15	ND ND	ND ND	ND ND	ND ND
16	2.31 (3.4)	21.18 (31.1)	7.97 (11.7)	11.03 (6.5)
17	3.51 (3.7)	34.54 (36.4)	22.11 (23.3)	25.81 (11.7)
18	3.50 (4.0)	35.26 (40.3)	25.14 (27.6)	20.56 (16.1)
19	4.65 (5.1)	40.77 (44.7)	28.39 (31.3)	22.44 (34.0)
20	4.82 (5.4)	41.57 (46.5)	30.40 (34.0)	30.57 (48.8)

FIGURE A 3.1 Cellular phenotype dynamics and parasite kinetics in peripheral afferent lymph from local skin reactions in sheep 669 infected with *T. congolense* TREU 1457

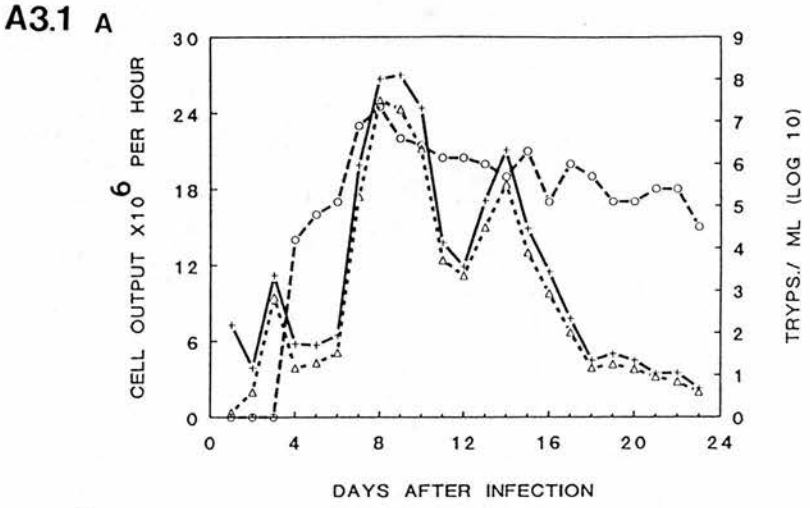
(A)
- Parasite kinetics
- Total cellular output
- Lymphocyte output

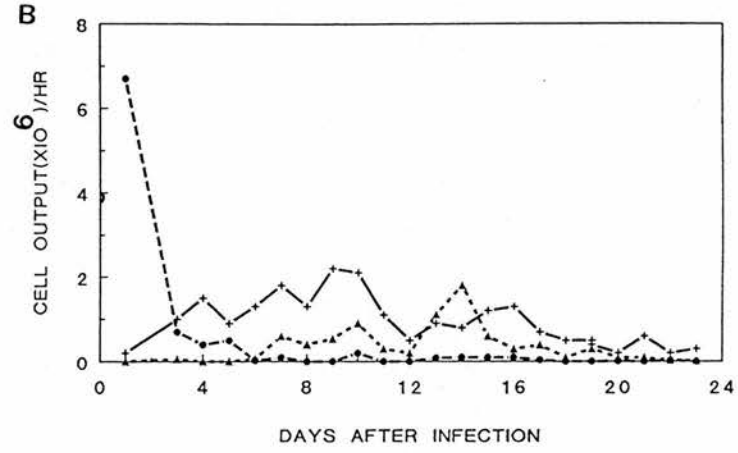
(B)
- Lymphoblasts
- Macrophages
- Granulocytes

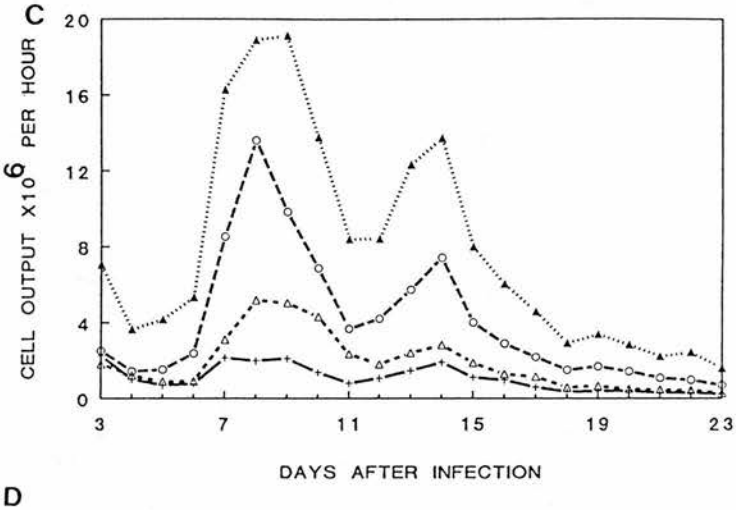
(C)
- $CD5^+$ cells
- $CD4^+$ cells
- $CD8^+$ cells
- SBU-$T19^+$ cells

(D)
- $CD45R^+$ cells
- SIg^+ cells
- $CD1^+$ cells
- MHC Class II^+ cells

A3.1


A
CELL OUTPUT X10 6 PER HOUR
TRYPS./ ML (LOG 10)
DAYS AFTER INFECTION


B
CELL OUTPUT(XIO 6)/HR
DAYS AFTER INFECTION


C
CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

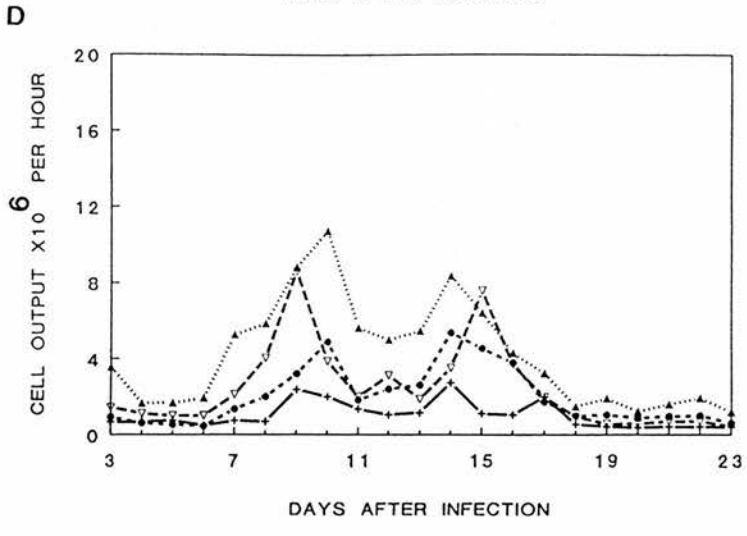

D
CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

TABLE A 3.5 Lymph flow, cellular responses and parasite kinetics in peripheral afferent lymph of sheep 669 after infection in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^6$)	Parasitosis (log_{10} tryps/ml)
1	44 (18 hrs)	3.3	7.3	0
2	72	1.3	3.9	0
3	158	1.7	11.2	0
4	126	1.1	5.8	4.2
5	130	1.06	5.7	4.8
6	130	1.2	6.5	5.1
7	141	3.4	19.9	6.9
8	123	5.2	26.7	7.35
9	120	5.4	27.0	6.6
10	122	4.8	24.4	6.45
11	114	2.9	13.8	6.15
12	106	2.7	11.9	6.15
13	105	3.9	17.1	6.0
14	92	5.5	21.1	5.7
15	94	3.8	14.9	6.3
16	95	2.9	11.5	5.1
17	124	1.5	7.8	6.0
18	49	2.2	4.5	5.7
19	108	1.1	5.0	5.1
20	64	1.7	4.5	5.1
21	104	0.8	3.5	5.4
22	106	0.8	3.5	5.4
23	90	0.6	2.3	4.5

TABLE A 3.6 Differential leucocyte counts of peripheral afferent lymph cells of sheep 669 infected with *T. congolense* TREU 1457. Absolute values (x10^6/hr) in brackets

Days after infection	Small lymphocytes(%)		Large lymphocytes(%)		Macrophages/ dendritic cells(%)		Granulocytes (%)	
1	5.0	(0.4)	0	(0)	3.0	(0.2)	92.0	(6.7)
2								
3	84.6	(9.5)	0.5	(0.06)	8.5	(1.0)	6.5	(0.7)
4	67.0	(3.9)	0	(0)	25.5	(1.5)	8.0	(0.4)
5	75.5	(4.3)	0	(0)	16.5	(0.9)	8.0	(0.5)
6	78.5	(5.1)	1.0	(0.07)	20.0	(1.3)	0.5	(0.03)
7	87.5	(17.4)	3.0	(0.6)	9.0	(1.8)	0.5	(0.1)
8	93.5	(25.0)	1.5	(0.4)	5.0	(1.3)	0	(0)
9	90.0	(24.3)	2.0	(0.54)	8.0	(2.2)	0	(0)
10	87.0	(21.2)	3.5	(0.9)	8.5	(2.1)	1.0	(0.2)
11	90.0	(12.4)	2.0	(0.3)	8.0	(1.1)	0	(0)
12	94.0	(11.2)	2.0	(0.2)	4.0	(0.5)	0	(0)
13	88.0	(15.0)	6.5	(1.11)	5.0	(0.9)	0.5	(0.09)
14	87.0	(18.4)	8.5	(1.8)	4.0	(0.8)	0.5	(0.1)
15	87.0	(13.0)	4.5	(0.6)	8.0	(1.2)	0.5	(0.1)
16	85.0	(9.8)	3.0	(0.3)	11.0	(1.3)	1.0	(0.1)
17	85.5	(6.7)	5.0	(0.39)	9.0	(0.7)	0.5	(0.04)
18	86.5	(3.9)	3.5	(0.1)	10.0	(0.5)	0	(0)
19	84.5	(4.2)	6.5	(0.3)	9.0	(0.5)	0	(0)
20	84.5	(3.8)	6.5	(0.3)	9.0	(0.4)	0	(0)
21	91.0	(3.2)	2.5	(0.09)	6.0	(0.2)	0.5	(0.01)
22	79.0	(2.8)	5.0	(0.1)	16.0	(0.6)	0	(0)
23	88.0	(2.0)	3.0	(0.03)	7.5	(0.2)	1.5	(0.03)

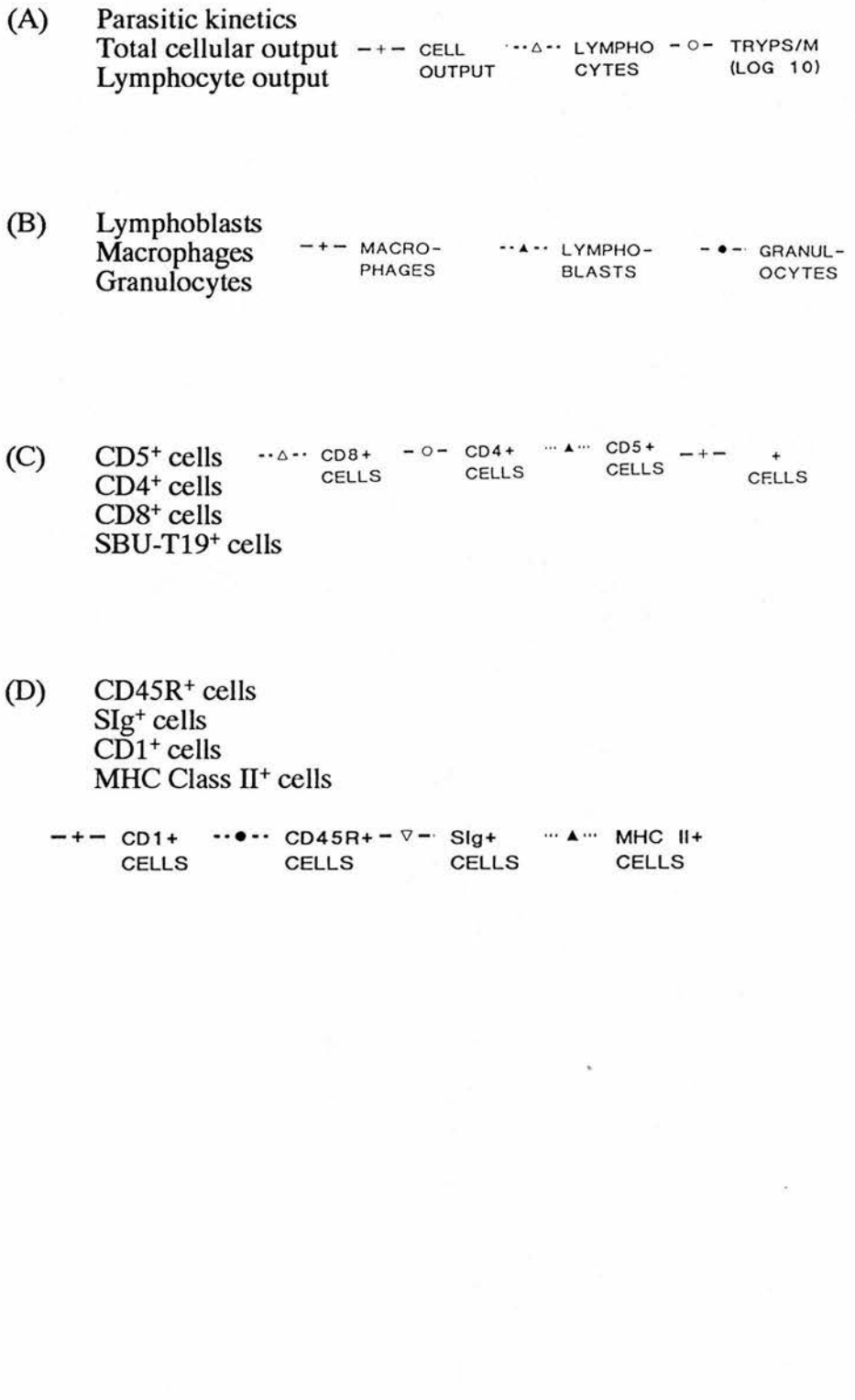
TABLE A 3.7 T lymphocyte subpopulation dynamics in peripheral afferent Lymph of sheep 669 infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
3	7.06 (63.0)	2.52 (22.5)	1.8 (16.1)	2.27 (20.3)
4	3.65 (62.9)	1.44 (24.9)	1.22 (21.0)	1.01 (17.4)
5	4.18 (73.3)	1.53 (26.8)	0.9 (15.8)	0.71 (12.4)
6	5.35 (82.3)	2.39 (36.8)	0.91 (14.0)	0.77 (11.9)
7	16.28 (81.8)	8.54 (42.9)	3.12 (15.7)	2.15 (10.8)
8	18.90 (70.8)	13.6 (50.9)	5.21 (19.5)	2.0 (7.5)
9	19.14 (70.9)	9.83 (36.4)	5.05 (18.7)	2.11 (7.8)
10	13.76 (56.4)	6.88 (28.2)	4.32 (17.7)	1.37 (5.6)
11	8.39 (60.8)	3.68 (26.7)	2.35 (17.0)	0.81 (5.9)
12	8.41 (70.7)	4.22 (35.5)	1.81 (15.2)	1.07 (9.0)
13	12.31 (72.0)	5.75 (33.6)	2.41 (14.1)	1.47 (8.6)
14	13.72 (65.0)	7.43 (35.2)	2.83 (13.4)	1.92 (9.1)
15	8.00 (53.7)	4.02 (27.0)	1.89 (12.7)	1.12 (7.5)
16	6.06 (52.7)	2.91 (25.3)	1.29 (11.2)	0.98 (8.5)
17	4.6 (59.0)	2.19 (28.1)	1.15 (14.8)	0.6 (7.7)
18	2.93 (65.0)	1.51 (33.5)	0.59 (13.0)	0.36 (8.0)
19	3.4 (67.9)	1.7 (34.0)	0.67 (13.4)	0.4 (8.0)
20	2.85 (63.4)	1.44 (32.0)	0.51 (11.4)	0.41 (9.2)
21	2.23 (63.8)	1.09 (31.0)	0.47 (13.3)	0.31 (8.8)
22	2.44 (69.8)	0.98 (27.9)	0.44 (12.6)	0.32 (9.1)
23	1.6 (69.5)	0.7 (30.5)	0.28 (12.2)	0.24 (10.3)

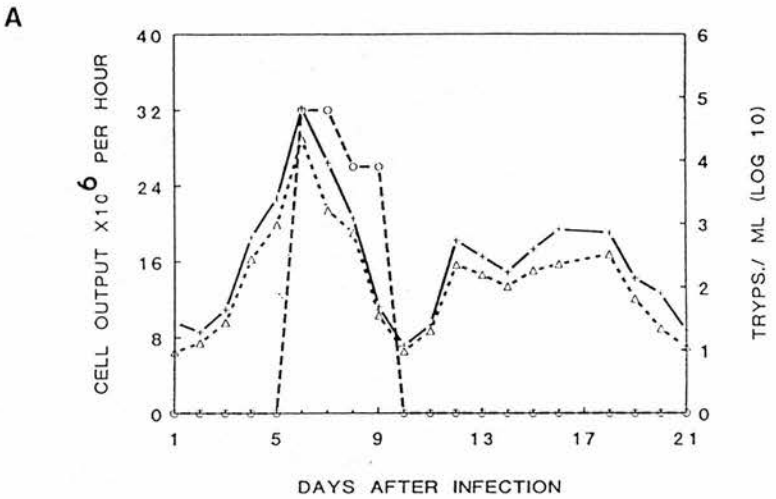
TABLE A 3.8 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in peripheral afferent lymph of sheep 669 infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

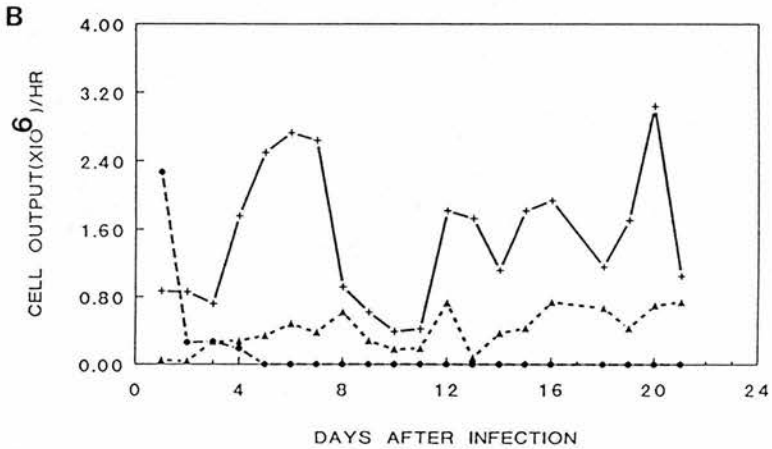
Days after infection	CD1$^+$ cells	MHC Class II$^+$ cells	CD45R$^+$ cells	SIg$^+$ cells
3	0.66 (5.9)	3.55 (31.7)	0.92 (8.2)	1.43 (12.8)
4	0.67 (11.6)	1.65 (28.4)	0.61 (10.5)	1.11 (19.2)
5	0.76 (13.4)	1.67 (29.3)	0.53 (9.3)	1.0 (17.6)
6	0.48 (7.4)	1.93 (29.7)	0.46 (7.0)	1.0 (15.4)
7	0.74 (3.7)	5.27 (26.5)	1.35 (6.8)	2.11 (10.6)
8	0.69 (2.6)	5.85 (21.9)	2.0 (7.5)	4.03 (15.1)
9	2.38 (8.8)	8.8 (32.6)	3.21 (11.9)	8.59 (31.8)
10	2.00 (8.2)	10.71 (43.9)	4.88 (20.0)	3.86 (15.8)
11	1.35 (9.8)	5.63 (40.8)	1.82 (13.2)	2.0 (14.5)
12	1.05 (8.8)	5.0 (42.0)	2.4 (20.2)	3.12 (26.2)
13	1.16 (6.8)	5.47 (32.0)	2.62 (15.3)	1.88 (11.0)
14	2.74 (13.0)	8.36 (39.6)	5.38 (25.5)	3.52 (16.7)
15	1.12 (7.5)	6.44 (43.2)	4.56 (30.6)	7.6 (51.0)
16	1.05 (9.1)	4.31 (37.5)	3.8 (33.0)	3.68 (32.9)
17	2.0 (25.6)	3.25 (41.7)	1.73 (22.2)	2.0 (25.7)
18	0.55 (12.3)	1.49 (33.1)	0.99 (22.1)	0.95 (21.1)
19	0.43 (8.6)	1.91 (38.1)	1.05 (20.9)	0.58 (11.6)
20	0.38 (8.4)	1.23 (27.4)	0.88 (19.6)	0.59 (13.0)
21	0.42 (11.9)	1.6 (45.8)	0.97 (27.7)	0.69 (19.6)
22	0.42 (12.0)	1.93 (55.0)	1.01 (28.9)	0.71 (20.3)
23	0.4 (17.5)	1.19 (51.6)	0.61 (26.5)	0.44 (19.3)

FIGURE A 3.2 Cellular phenotype dynamics and parasite kinetics in peripheral afferent lymph from local skin reactions in sheep 680L infected with *T. congolense* TREU 1881



A3.2


A
CELL OUTPUT X10 6 PER HOUR
TRYPS./ ML (LOG 10)
DAYS AFTER INFECTION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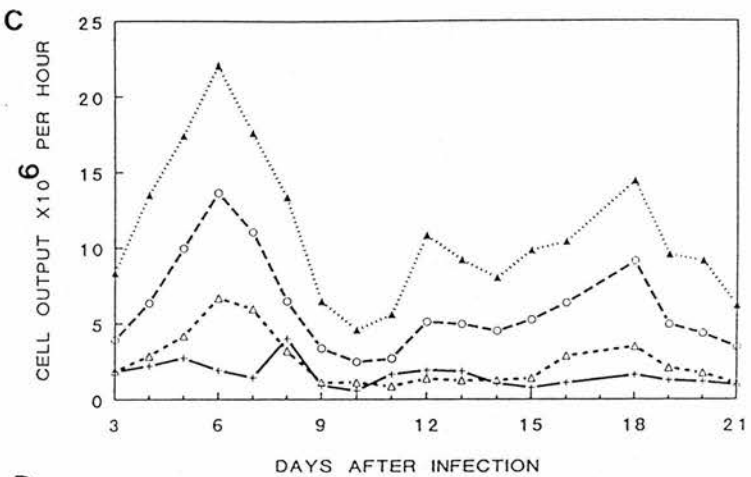
CELL OUTPUT(X10 6)/HR
DAYS AFTER INFECTION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CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

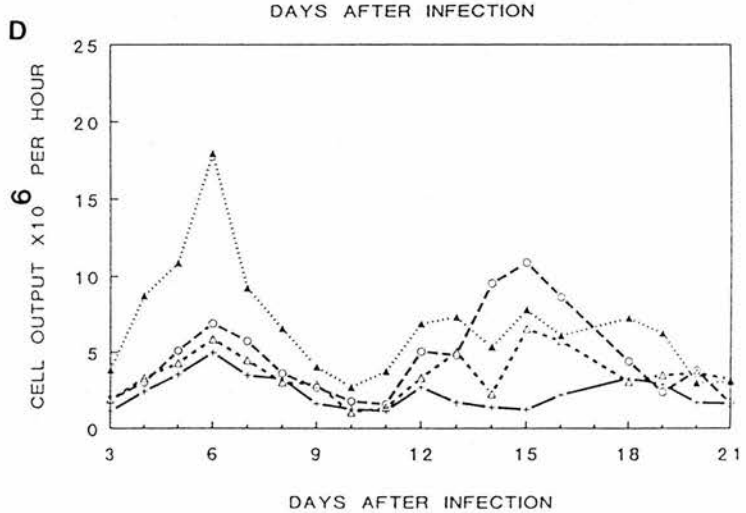

D
CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

TABLE A 3.9 Lymph flow, cellular responses and parasite kinetics in peripheral afferent lymph of sheep 680L after infection in drainage area with *T. congolense* TREU 1881

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^6$)	Parasitosis (log_{10} tryps/ml)
1	92 (18 hrs)	2.1	9.66	0
2	172	1.2	8.58	0
3	202	1.3	10.96	0
4	212	2.1	18.54	0
5	188	2.9	22.71	0
6	227	3.4	32.17	4.8
7	198	3.2	26.42	4.8
8	170	2.9	20.54	3.9
9	180	1.5	11.25	3.9
10	90	1.9	7.13	0
11	160	1.4	9.33	0
12	162	2.7	18.21	0
13	165	2.4	16.5	0
14	115	3.1	14.88	0
15	122	3.4	17.29	0
16	150	3.1	19.38	0
17	- -	-	0	
18	152	3.0	19.0	0
19	180	1.9	14.25	0
20	190	1.6	12.67	0
21	210	1.0	8.75	0

TABLE A 3.10 Differential leucocyte counts of peripheral lymph cells of sheep 680L infected with *T. congolense* TREU 1881. Absolute values (x10^6/hr) in brackets

Days after infection	Small lymphocytes(%)	Large lymphocytes(%)	Macrophages dendritic cells(%)	Granulocytes (%)
1	67.0 (6.47)	0.5 (0.05)	9.0 (0.87)	23.5 (2.27)
2	86.5 (7.42)	0.5 (0.04)	10.0 (0.86)	3.0 (0.26)
3	87.5 (9.59)	2.5 (0.27)	7.5 (0.72)	2.5 (0.27)
4	88.0 (16.24)	1.5 (0.28)	9.5 (1.76)	1.0 (0.19)
5	87.5 (19.87)	1.5 (0.34)	11.0 (2.5)	0 (0)
6	90.0 (28.95)	1.5 (0.48)	8.5 (2.73)	0 (0)
7	81.0 (21.4)	9.0 (0.38)	10.0 (2.64)	0 (0)
8	92.5 (19.0)	3.0 (0.62)	4.5 (0.92)	0 (0)
9	92.0 (10.35)	2.5 (0.28)	5.5 (0.62)	0 (0)
10	92.0 (6.56)	2.5 (0.18)	5.5 (0.39)	0 (0)
11	93.5 (8.72)	2.0 (0.19)	4.5 (0.42)	0 (0)
12	86.0 (15.66)	4.0 (0.73)	10.0 (1.82)	0 (0)
13	88.5 (14.6)	0.5 (0.08)	10.5 (1.73)	0 (0)
14	90.0 (13.39)	2.5 (0.37)	7.5 (1.12)	0 (0)
15	87.0 (15.04)	2.5 (0.43)	10.5 (1.82)	0 (0)
16	81.0 (15.7)	9.0 (0.74)	10.0 (1.94)	0 (0)
17	- -	- -	- -	- -
18	88.0 (16.72)	3.5 (0.67)	8.5 (1.16)	0 (0)
19	85.0 (12.11)	3.0 (0.43)	12.0 (1.71)	0 (0)
20	70.5 (8.93)	5.5 (0.7)	24.0 (3.04)	0 (0)
21	79.5 (6.96)	8.5 (0.74)	12.0 (1.05)	0 (0)

TABLE A 3.11 T lymphocyte subpopulation dynamics in peripheral afferent lymph of sheep 680L infected with *T. congolense* TREU 1881. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
3	8.35 (76.2)	3.92 (35.8)	1.89 (17.2)	1.85 (16.9)
4	13.5 (72.8)	6.36 (34.3)	2.86 (15.4)	2.22 (12.0)
5	17.4 (76.6)	9.95 (43.8)	4.18 (18.4)	2.73 (12.0)
6	22.04 (68.5)	13.64 (42.4)	6.69 (20.8)	1.93 (6.0)
7	17.6 (66.6)	11.04 (41.8)	5.97 (22.6)	1.45 (5.5)
8	13.35 (65.0)	6.49 (31.6)	3.2 (15.6)	4.01 (19.5)
9	6.47 (57.5)	3.36 (29.9)	1.13 (10.0)	0.92 (8.2)
10	4.58 (64.3)	2.48 (34.8)	1.12 (15.7)	0.58 (8.1)
11	5.6 (6.13)	2.7 (28.9)	0.87 (9.3)	1.66 (17.8)
12	10.82 (59.4)	5.12 (28.1)	1.37 (7.5)	1.93 (10.6)
13	9.21 (55.8)	4.97 (30.1)	1.25 (7.6)	1.86 (11.3)
14	8.05 (54.1)	4.51 (30.3)	1.26 (8.5)	1.05 (7.8)
15	9.84 (56.9)	5.27 (30.5)	1.4 (8.1)	0.78 (4.5)
16	10.41 (53.7)	6.38 (32.9)	2.87 (14.8)	1.1 (5.7)
17	- -	- -	- -	- -
18	14.44 (76.0)	9.14 (48.1)	3.5 (18.4)	1.63 (8.6)
19	9.55 (67.0)	4.96 (34.8)	2.09 (14.7)	1.27 (8.9)
20	9.17 (72.4)	4.36 (34.4)	1.75 (13.8)	1.18 (9.3)
21	6.23 (71.2)	3.46 (39.5)	1.06 (12.1)	0.96 (11.0)

TABLE A 3.12 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in peripheral afferent lymph of sheep 680L infected with *T. congolense* TREU 1881. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

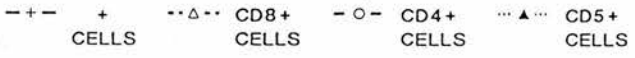
Days after infection	CD1$^+$ cells	MHC Class II$^+$ cells	CD45R$^+$ cells	SIg$^+$ cells
3	1.14 (10.4)	3.84 (35.0)	1.89 (17.2)	1.89 (17.2)
4	2.43 (13.1)	8.73 (47.1)	3.32 (17.9)	3.02 (16.3)
5	3.54 (15.6)	10.86 (47.8)	4.29 (18.9)	5.11 (22.5)
6	4.99 (15.5)	17.95 (55.8)	5.89 (18.3)	6.92 (21.5)
7	3.51 (13.3)	9.25 (35.0)	4.49 (17.0)	5.76 (21.8)
8	3.25 (15.8)	6.59 (32.1)	3.04 (14.8)	3.62 (17.6)
9	1.63 (14.5)	4.05 (36.0)	2.90 (25.8)	2.68 (23.8)
10	1.28 (18.0)	2.71 (38.0)	1.06 (14.9)	1.8 (25.3)
11	1.16 (12.4)	3.76 (40.3)	1.38 (14.8)	1.59 (17.0)
12	2.71 (14.9)	6.9 (37.9)	3.33 (18.3)	5.06 (27.8)
13	1.72 (10.4)	7.34 (44.5)	5.05 (30.6)	4.83 (29.3)
14	1.4 (9.4)	5.37 (36.1)	2.26 (15.2)	9.55 (64.2)
15	1.24 (7.2)	7.83 (45.3)	6.57 (38.0)	10.91 (63.1)
16	2.19 (11.3)	6.14 (31.7)	5.77 (29.8)	8.66 (44.7)
17	- -	- -	- -	- -
18	3.31 (17.4)	7.28 (38.3)	3.08 (16.2)	4.43 (23.3)
19	2.89 (20.3)	6.28 (44.1)	3.51 (24.6)	2.38 (16.7)
20	1.7 (13.4)	2.98 (23.5)	3.65 (28.8)	3.8 (36.0)
21	1.65 (18.8)	3.07 (35.1)	3.23 (36.9)	1.65 (18.8)

FIGURE A 3.3 Cellular phenotype dynamics and parasite kinetics in peripheral afferent lymph from local skin reactions in sheep 680R infected with *T. congolense* TREU 1457

(A) Parasite kinetics
Total cellular output
Lymphocyte output

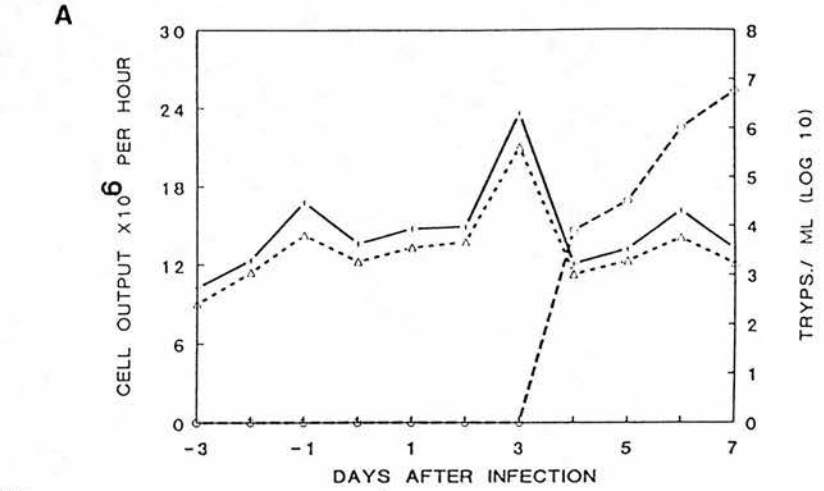
(B) Lymphoblasts
Macrophages
Granulocytes

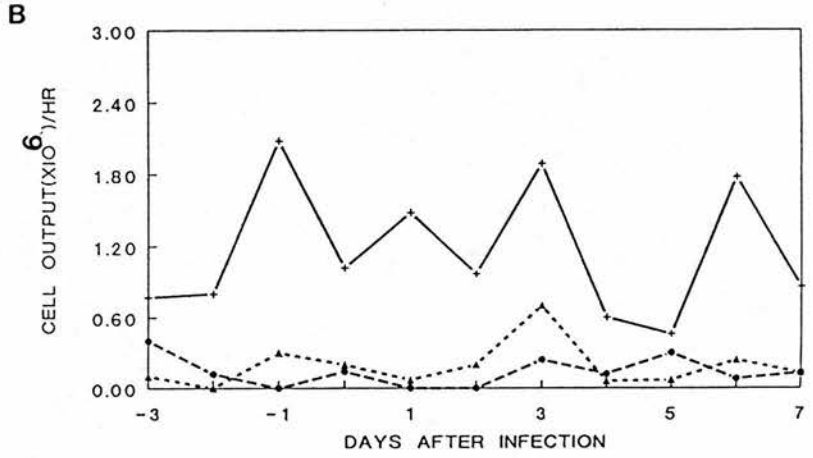
(C) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

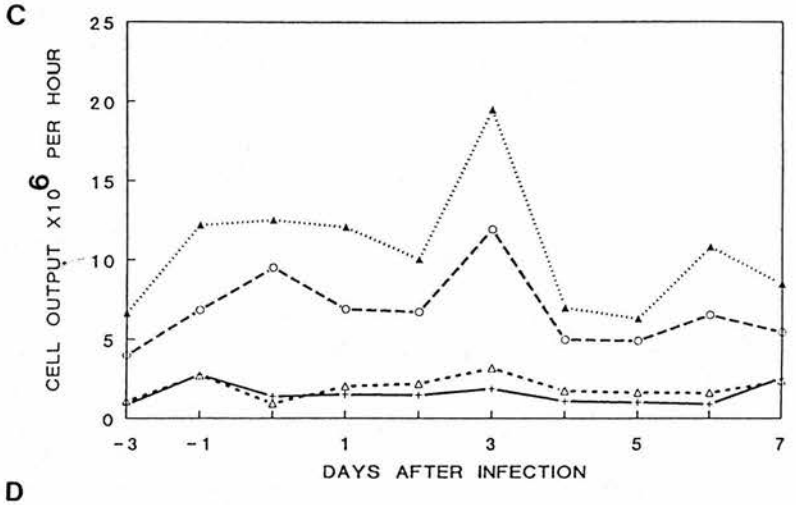


(D) $CD45R^+$ cells
SIg^+ cells
$CD1^+$ cells
MHC Class II^+ cells

A3.3


A
CELL OUTPUT X10 6 PER HOUR
TRYPS./ ML (LOG 10)
DAYS AFTER INFECTION


B
CELL OUTPUT(XIO 6)/HR
DAYS AFTER INFECTION


C
CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

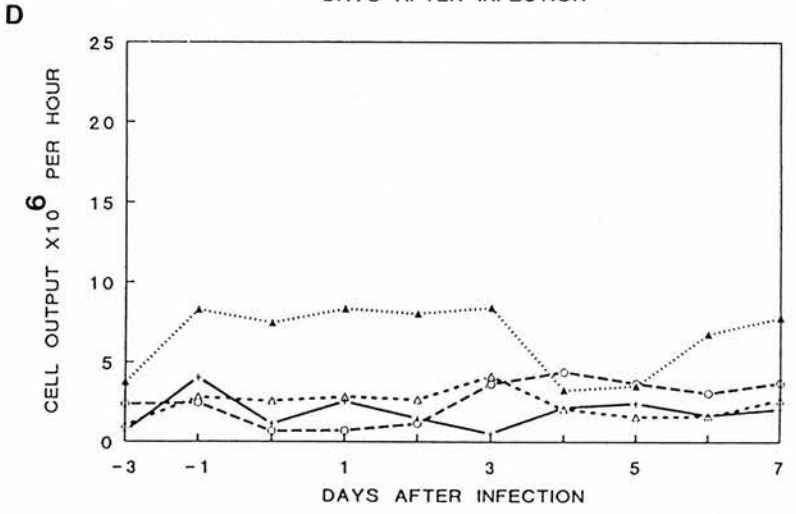

D
CELL OUTPUT X10 6 PER HOUR
DAYS AFTER INFECTION

TABLE A 3.13 Lymph flow, cellular responses and parasite kinetics in peripheral afferent lymph of sheep 680R after infection in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^6$)	Parasitosis (log_{10} tryps/ml)
-5	116 (18 hrs)	-	-	-
-4	137	-	-	-
-3	112	2.2	10.27	-
-2	174	1.7	12.33	-
-1	250	1.6	16.77	-
0	272	1.2	13.6	-
1	236	1.5	14.75	0
2	179	2.0	14.92	0
3	172	3.3	23.65	0
4	126	2.3	12.08	0
5	155	2.4	13.2	4.5
6	155	2.5	16.15	6.0
7	198	1.6	13.2	6.75

TABLE A 3.14 Differential leucocyte counts of peripheral afferent lymph cells of sheep 680R infected with *T. congolense* TREU 1457. Absolute values (x10^6/hr) in brackets

Days after infection	Small lymphocytes(%)	Large lymphocytes(%)	Macrophages/ dendritic cells(%)	Granulocytes (%)
-3	88.0 (9.04)	1.0 (0.1)	7.5 (0.77)	3.5 (0.4)
-2	92.5 (11.41)	0 (0)	6.5 (0.80)	1.0 (0.12)
-1	85.5 (14.25)	2.0 (0.3)	12.5 (2.08)	0 (0)
0	90.0 (12.24)	1.5 (0.2)	7.5 (1.02)	1.0 (0.14)
1	89.5 (13.29)	0.5 (0.07)	10.0 (1.48)	0 (0)
2	92.0 (13.73)	1.5 (0.2)	6.5 (0.97)	0 (0)
3	89.0 (21.05)	2.0 (0.7)	8.0 (1.89)	1.0 (0.24)
4	93.5 (11.29)	0.5 (0.06)	5.0 (0.6)	1.0 (0.12)
5	93.0 (12.28)	0.5 (0.07)	3.5 (0.46)	3.0 (0.3)
6	87.0 (14.05)	1.5 (0.24)	11.0 (1.78)	0.5 (0.08)
7	91.5 (12.08)	1.0 (0.13)	6.5 (0.86)	1.0 (0.13)

TABLE A 3.15 T lymphocyte subpopulation dynamics in peripheral afferent lymph of sheep infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

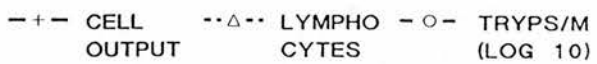
Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	$\tau\delta^+$TcR cells
-3	6.64 (64.5)	3.99 (38.7)	1.11 (10.8)	0.84 (8.2)
-2	- -	- -	- -	- -
-1	12.19 (73.1)	6.85 (41.1)	2.72 (16.3)	2.77 (16.6)
0	12.50 (91.9)	9.51 (69.9)	0.94 (6.9)	1.4 (10.3)
1	12.05 (81.7)	6.9 (46.8)	2.04 (13.8)	1.52 (10.3)
2	10.03 (67.2)	6.73 (45.1)	2.21 (14.8)	1.49 (10.0)
3	19.46 (82.3)	11.92 (50.4)	3.19 (13.5)	1.89 (8.0)
4	6.99 (57.9)	4.98 (41.3)	1.75 (14.5)	1.09 (9.0)
5	6.31 (47.8)	4.91 (37.2)	1.65 (12.5)	1.09 (7.7)
6	10.84 (67.1)	6.56 (40.6)	1.62 (10.0)	0.9 (5.6)
7	8.51 (64.5)	5.45 (41.3)	2.4 (18.2)	2.56 (19.4)

TABLE A 3.16 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in peripheral afferent lymph of sheep 680R infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	CD1$^+$ cells	MHC Class II$^+$ cells	CD45R$^+$ cells	SIg$^+$ cells
-3	0.6 (5.8)	3.73 (36.2)	1.03 (10.0)	2.37 (23.0)
-2	- -	- -	- -	- -
-1	4.03 (24.2)	8.3 (49.8)	2.8 (16.8)	2.45 (14.7)
0	1.13 (8.3)	7.48 (55.0)	2.57 (18.9)	0.67 (4.9)
1	2.54 (17.2)	8.36 (56.7)	2.83 (19.2)	0.71 (4.8)
2	1.58 (9.9)	8.06 (54.0)	2.66 (17.8)	1.15 (7.7)
3	0.52 (2.2)	8.44 (35.7)	4.14 (17.5)	3.64 (15.4)
4	2.16 (17.9)	3.25 (26.9)	2.05 (17.0)	4.4 (36.4)
5	2.42 (18.3)	3.51 (26.6)	1.57 (11.9)	3.68 (27.9)
6	1.68 (10.4)	6.8 (42.1)	1.62 (10.0)	3.07 (19.0)
7	2.07 (15.7)	7.83 (59.3)	2.65 (20.1)	3.71 (28.1)

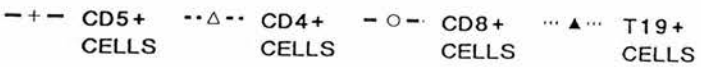
FIGURE A 3.4 Cellular phenotype dynamics and parasite kinetics in peripheral afferent lymph of sheep 680 following homologous challenge with *T. congolense* TREU 1457

(A) Parasite kinetics
Total cellular output
Lymphocyte output

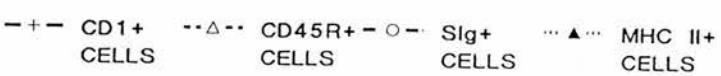


(B) Lymphoblasts
Macrophages
Granulocytes

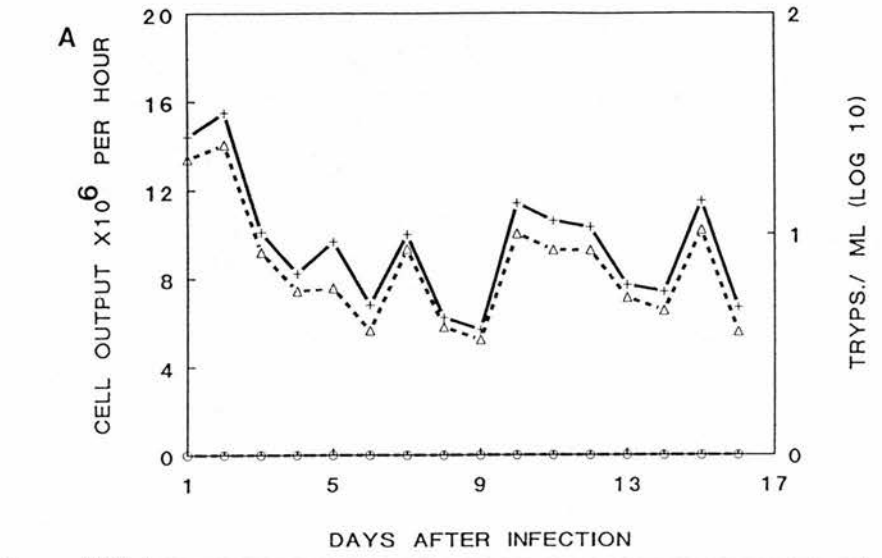
(C) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

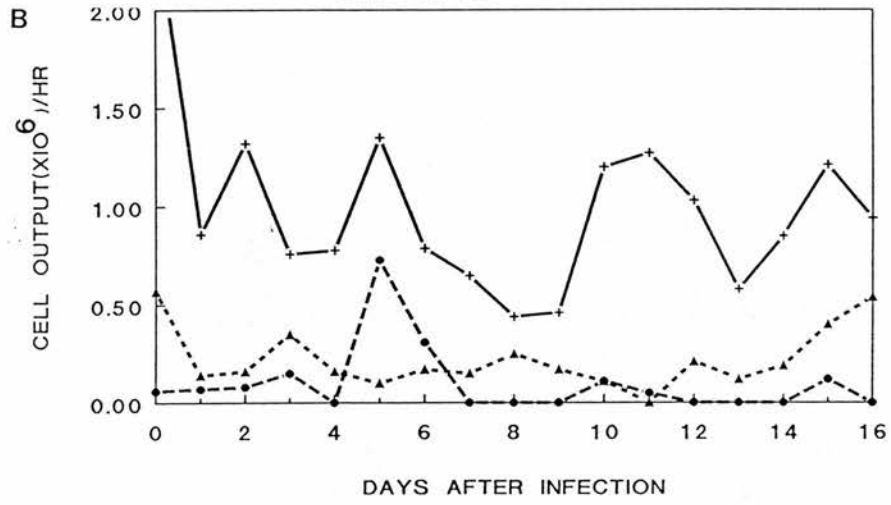


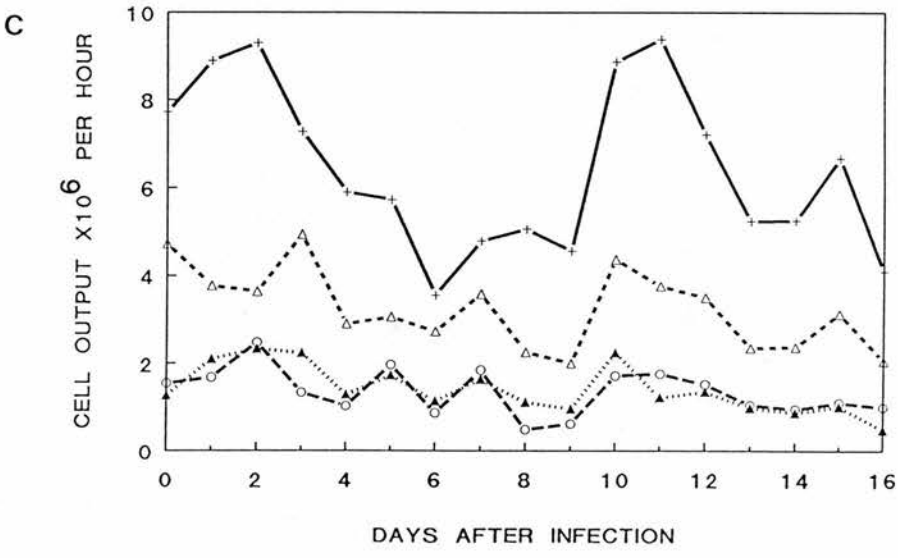
(D) $CD45R^+$ cells
SIg^+ cells
$CD1^+$ cells
MHC Class II^+ cells



A3.4


A
CELL OUTPUT X10^6 PER HOUR
TRYPS./ ML (LOG 10)
DAYS AFTER INFECTION


B
CELL OUTPUT(X10^6)/HR
DAYS AFTER INFECTION


C
CELL OUTPUT X10^6 PER HOUR
DAYS AFTER INFECTION

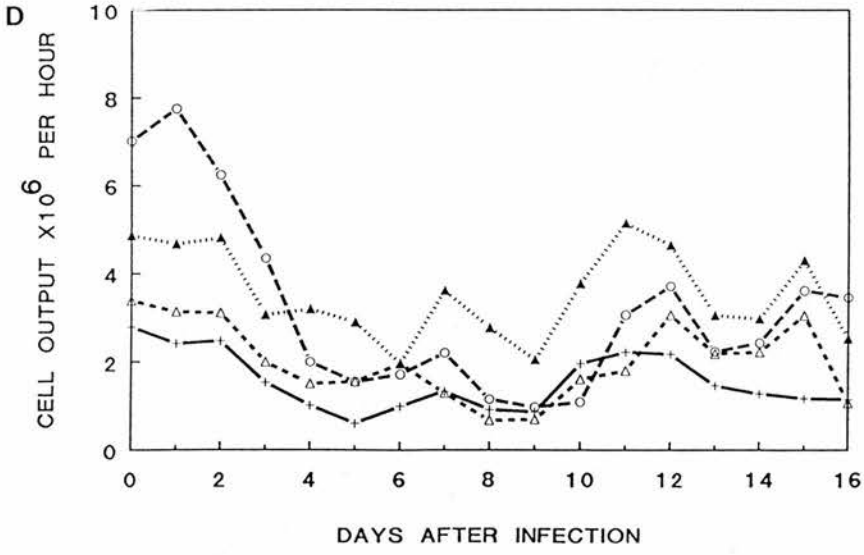

D
CELL OUTPUT X10^6 PER HOUR
DAYS AFTER INFECTION

TABLE A 3.17 Lymph flow, cellular responses and parasite kinetics in peripheral (afferent) lymph of sheep 680 after treatment and challenge in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^6$)	Parasitosis (log_{10} tryps/ml)
0	160	1.9	12.7	-
1	150	2.3	14.4	-
2	128	2.9	15.5	-
3	110	2.2	10.1	-
4	105	1.9	8.23	-
5	115	1.6	9.67	-
6	117	1.4	6.83	-
7	160	1.5	10.00	-
8	125	1.2	6.25	-
9	114	1.5	5.70	-
10	182	1.7	11.4	-
11	150	1.9	10.6	-
12	130	1.3	10.3	-
13	143	1.7	7.7	-
14	105	1.7	7.4	-
15	162	1.7	11.5	-
16	80	2.0	6.7	-

TABLE A 3.18 Differential leucocyte counts of peripheral (afferent) lymph cells of sheep 680 after treatment and challenge with *T. congolense* TREU 1457. Absolute values (x10^6/hr) in brackets

Days after infection	Small lymphocytes(%)	Large lymphocytes(%)	Macrophages/ dendritic cells(%)	Granulocytes (%)
0	75.5 (9.59)	4.5 (0.57)	19.5 (2.48)	0.5 (0.06)
1	92.5 (13.3)	1.0 (0.14)	6.0 (0.86)	0.5 (0.07)
2	90.0 (13.9)	1.0 (0.16)	8.5 (1.32)	0.5 (0.08)
3	87.5 (8.84)	3.5 (0.35)	7.5 (0.76)	1.5 (0.15)
4	88.5 (7.28)	2.0 (0.16)	9.5 (0.78)	0 TCW(0)
5	77.5 (7.49)	1.0 (0.10)	14.0 (1.35)	7.5 (0.73)
6	80.5 (5.50)	2.5 (0.17)	11.5 (0.79)	4.5 (0.31)
7	92.0 (9.20)	1.5 (0.15)	6.5 (0.65)	0 (0)
8	89.0 (5.56)	4.0 (0.25)	7.0 (0.44)	0 (0)
9	89.0 (5.07)	3.0 (0.17)	8.0 (0.46)	0 (0)
10	87.5 (9.98)	1.0 (0.11)	10.5 (1.20)	1.0 (0.11)
11	87.5 (9.28)	0.0 (0)	12.0 (1.27)	0.5 (0.05)
12	88.0 (9.06)	2.0 (0.21)	10.0 (1.03)	0 (0)
13	91.0 (7.01)	1.5 (0.12)	7.5 (0.58)	0 (0)
14	86.0 (6.36)	2.5 (0.19)	11.5 (0.85)	0 (0)
15	85.0 (9.78)	3.5 (0.40)	10.5 (1.21)	1.0 (0.12)
16	77.0 (5.16)	8.0 (0.54)	14.0 (0.94)	0.0 (0)

TABLE A 3.19 T lymphocyte subpopulation dynamics in peripheral (afferent) lymph of sheep infected with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
0	7.72 (60.8)	4.72 (37.2)	1.55 (12.2)	1.27 (10.0)
1	8.89 (61.7)	3.77 (26.2)	1.68 (11.7)	2.10 (14.6)
2	9.30 (60.0)	3.64 (23.5)	2.48 (16.0)	2.33 (15.0)
3	7.28 (72.1)	4.94 (48.9)	1.34 (13.3)	2.24 (22.2)
4	5.90 (71.7)	2.90 (35.2)	1.03 (12.5)	1.29 (15.7)
5	5.73 (59.3)	3.06 (31.6)	1.97 (20.4)	1.73 (17.9)
6	3.56 (52.1)	2.73 (39.9)	0.87 (12.7)	1.14 (16.7)
7	4.79 (47.9)	3.59 (35.9)	1.85 (18.5)	1.64 (16.4)
8	5.06 (81.0)	2.26 (36.2)	0.49 (7.9)	1.11 (17.8)
9	4.56 (80.0)	2.00 (35.0)	0.62 (10.8)	0.96 (16.8)
10	8.87 (77.8)	4.37 (38.3)	1.72 (15.1)	2.24 (19.7)
11	9.38 (88.5)	3.76 (35.5)	1.76 (16.6)	1.22 (11.6)
12	7.21 (70.0)	3.50 (34.0)	1.52 (14.8)	1.35 (13.1)
13	5.24 (68.0)	2.35 (30.5)	1.05 (13.7)	0.98 (12.7)
14	5.25 (71.0)	2.37 (32.0)	0.95 (12.9)	0.87 (11.8)
15	6.67 (58.0)	3.13 (27.2)	1.10 (9.6)	1.01 (8.8)
16	4.09 (61.1)	2.05 (30.6)	1.00 (14.9)	0.48 (7.1)

TABLE A 3.20 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in peripheral (afferent) lymph of sheep 680 after treatment and challenge with *T. congolense* TREU 1457. Absolute cell output x10^6/hr and relative proportions (percent fluorescent cells)

Days after infection	CD1$^+$ cells	MHC Class II$^+$ cells	CD45R$^+$ cells	SIg$^+$ cells
0	2.79 (22.0)	4.86 (38.3)	3.39 (26.7)	7.02 (55.3)
1	2.42 (16.8)	4.67 (32.4)	3.14 (21.8)	7.76 (53.9)
2	2.48 (16.0)	4.81 (31.0)	3.12 (20.1)	6.25 (40.3)
3	1.54 (15.2)	3.07 (30.4)	2.00 (19.8)	4.35 (43.1)
4	1.02 (12.4)	3.20 (38.9)	1.51 (18.4)	2.00 (24.2)
5	0.61 (6.3)	2.90 (30.0)	1.56 (16.1)	1.55 (16.0)
6	1.00 (14.6)	1.97 (28.8)	1.96 (28.7)	1.72 (25.2)
7	1.34 (13.4)	3.62 (36.2)	1.30 (13.0)	2.21 (22.1)
8	0.92 (14.7)	2.78 (44.5)	0.68 (10.9)	1.16 (18.5)
9	0.87 (15.2)	2.05 (36.0)	0.70 (12.3)	0.98 (17.2)
10	1.96 (17.2)	3.77 (33.1)	1.61 (14.1)	1.09 (9.6)
11	2.22 (20.9)	5.14 (48.5)	1.80 (17.0)	3.06 (28.9)
12	2.17 (21.1)	4.64 (45.0)	3.06 (29.7)	3.71 (36.0)
13	1.46 (18.9)	3.05 (39.6)	2.18 (28.3)	2.23 (28.9)
14	1.28 (17.3)	2.97 (40.1)	2.22 (30.0)	2.43 (32.8)
15	1.17 (10.2)	4.29 (37.3)	3.05 (26.5)	3.61 (31.4)
16	1.15 (17.2)	2.52 (37.6)	1.07 (15.9)	3.46 (51.6)

APPENDIX IV
PREFEMORAL EFFERENT LYMPHATIC CANNULATION DATA

TABLE E 4.1 Lymph flow, cellular responses and parasite kinetics in prefemoral efferent lymph of sheep 758 after infection in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output(10^7)	Parasitosis (log_{10} tryps/ml)	Lymphoblasts ($x10^7$/hr) (%)
-5	133 (18 hrs)	-	-	-	-
-4	176	-	-	-	-
-3	200	10.8	9.0	-	-
-2	120	16.6	8.3	-	0.46 (5.5)
-1	220	14.0	12.8	-	0.19 (1.5)
0	174	10.6	7.7	-	0.08 (1.0)
1	155	13.1	8.5	-	0.17 (2.0)
2	148	12.4	7.6	-	0.27 (3.5)
3	90	16.7	6.3	-	0.35 (5.5)
4	130	17.0	9.2	-	0.37 (4.0)
5	132	16.8	9.2	-	0.51 (5.5)
6	104	27.5	11.9	4.8	2.08 (17.5)
7	120	42.9	21.5	5.4	10.21 (47.5)
8	100	69.9	29.1	6.0	8.15 (28.0)
9	75	73.6	23.0	6.45	11.62 (50.5)
10	85	40.1	14.2	6.3	5.04 (35.5)
11	104	30.9	13.4	5.7	3.22 (24.0)
12	85	35.6	12.6	6.45	2.52 (20.0)
13	58	46.9	11.3	5.4	3.16 (28.0)
14	58	51.5	12.4	5.1	1.49 (12.0)
15	54	60.8	13.7	4.8	3.08 (22.5)
16	66	26.6	7.3	4.8	0.58 (8.0)
17	78	27.7	9.0	4.5	1.13 (12.5)
18	81	24.0	8.1	0	1.01 (12.5)
19	132	10.6	5.8	5.7	1.94 (33.5)
20	77	14.9	4.8	5.7	0.55 (11.5)
21	100	14.2	5.9	4.5	0.53 (9.0)

TABLE E 4.2 T lymphocyte subpopulation dynamics in prefemoral efferent lymph of sheep 758 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
0	6.34 (82.3)	3.98 (51.7)	1.39 (18.0)	0.42 (5.4)
1	7.36 (86.6)	4.98 (58.6)	1.57 (18.5)	0.37 (4.4)
2	6.04 (79.5)	4.10 (53.9)	1.24 (16.3)	0.38 (5.0)
3	5.22 (83.0)	3.33 (52.9)	1.07 (17.0)	0.26 (4.2)
4	7.27 (79.0)	5.36 (58.3)	1.67 (18.2)	0.20 (2.2)
5	7.31 (79.5)	6.08 (66.1)	1.27 (13.8)	0.36 (3.9)
6	8.84 (74.3)	6.58 (55.3)	1.87 (15.7)	0.45 (3.8)
7	12.47 (58.0)	9.63 (44.8)	3.68 (17.1)	1.33 (6.2)
8	13.88 (47.7)	8.79 (30.2)	3.32 (11.4)	0.35 (1.2)
9	11.36 (49.4)	8.10 (35.2)	2.97 (12.9)	0.41 (1.8)
10	8.97 (63.2)	6.29 (44.3)	1.72 (12.1)	0.26 (1.8)
11	9.09 (67.8)	6.18 (46.1)	1.45 (10.8)	0.24 (1.8)
12	7.51 (59.6)	5.54 (44.0)	1.10 (8.7)	0.32 (2.5)
13	5.55 (49.1)	4.18 (37.0)	1.18 (10.4)	0.41 (3.6)
14	4.99 (40.3)	3.32 (26.8)	1.07 (8.6)	0.29 (2.3)
15	5.21 (38.0)	4.00 (29.2)	1.44 (10.4)	0.42 (3.1)
16	4.23 (58.0)	3.11 (42.6)	1.11 (15.2)	0.45 (6.1)
17	5.24 (58.2)	3.65 (40.5)	1.63 (18.1)	0.47 (5.2)
18	4.47 (55.2)	3.22 (39.7)	1.56 (19.3)	0.55 (6.8)
19	3.10 (53.5)	1.94 (33.4)	0.75 (12.9)	0.16 (2.8)
20	2.89 (60.3)	1.87 (39.0)	0.80 (16.6)	0.17 (3.5)
21	3.93 (66.7)	2.63 (44.5)	0.87 (14.8)	0.26 (4.4)

TABLE E 4.3 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in prefemoral efferent lymph of sheep 758 infected with *T. congolense* TREU 1457. Absolute cells output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	MHC Class II$^+$ (SW73.2) cells	MHC Class II$^+$ (SBU-II) cells	CD45R$^+$ cells	SIg$^+$ cells
0	1.18 (15.3)	1.01 (13.1)	1.96 (25.5)	0.63 (8.2)
1	1.05 (12.3)	1.31 (15.4)	1.14 (13.4)	1.33 (15.6)
2	1.19 (15.7)	0.81 (10.6)	0.92 (12.1)	1.25 (16.5)
3	1.24 (19.7)	0.85 (13.5)	0.93 (14.8)	1.31 (20.8)
4	0.93 (10.1)	1.44 (15.6)	0.75 (8.1)	1.42 (15.4)
5	1.27 (13.8)	1.83 (19.9)	1.98 (21.5)	1.42 (15.4)
6	3.36 (28.2)	3.32 (27.9)	3.43 (28.8)	4.37 (36.7)
7	5.91 (27.5)	7.68 (35.7)	9.27 (43.1)	11.00 (51.2)
8	9.84 (33.8)	12.69 (43.6)	16.12 (55.4)	20.28 (69.7)
9	11.13 (48.4)	10.51 (45.7)	11.41 (49.6)	13.43 (58.4)
10	5.06 (35.6)	3.21 (22.6)	4.08 (28.7)	2.57 (18.1)
11	5.55 (41.4)	4.23 (31.6)	4.61 (34.4)	3.79 (28.3)
12	5.20 (41.3)	4.99 (39.6)	4.57 (36.3)	3.69 (29.3)
13	4.86 (43.0)	4.63 (41.0)	4.14 (36.6)	7.11 (62.9)
14	6.41 (51.7)	5.78 (46.6)	6.81 (54.9)	7.82 (63.1)
15	9.18 (67.0)	4.96 (36.2)	4.67 (34.1)	8.62 (62.9)
16	4.20 (57.5)	3.04 (41.6)	3.57 (48.9)	4.72 (64.7)
17	4.91 (54.5)	3.58 (39.8)	4.04 (44.9)	4.08 (45.3)
18	3.31 (40.9)	3.38 (41.7)	3.35 (41.4)	4.33 (53.4)
19	2.91 (50.2)	2.44 (42.0)	3.05 (52.6)	1.90 (32.8)
20	1.90 (39.5)	1.47 (30.6)	2.02 (42.0)	1.52 (31.6)
21	1.68 (28.4)	1.55 (26.2)	1.85 (31.4)	2.34 (39.6)

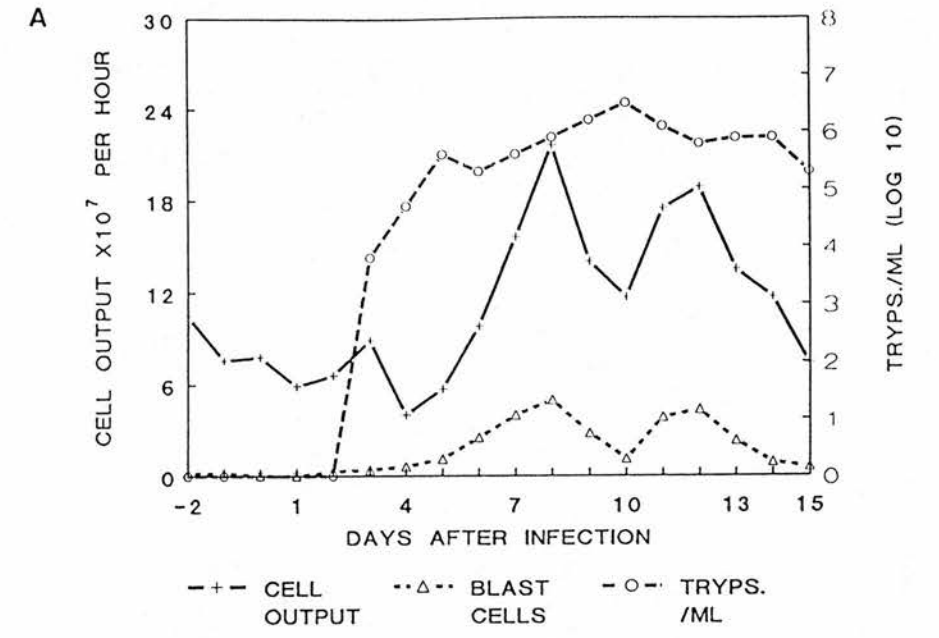
FIGURE E 4.1 Cellular phenotype dynamics and parasite kinetics in prefemoral efferent lymph from lymph nodes draining local skin reactions in sheep 749 infected with *T. congolense* TREU 1457

(A) Parasite kinetics
Total cellular output
Lymphoblast output

(B) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

(C) SIg^+ cells
$CD45R^+$ cells
MHC Class II^+ cells

E 4.1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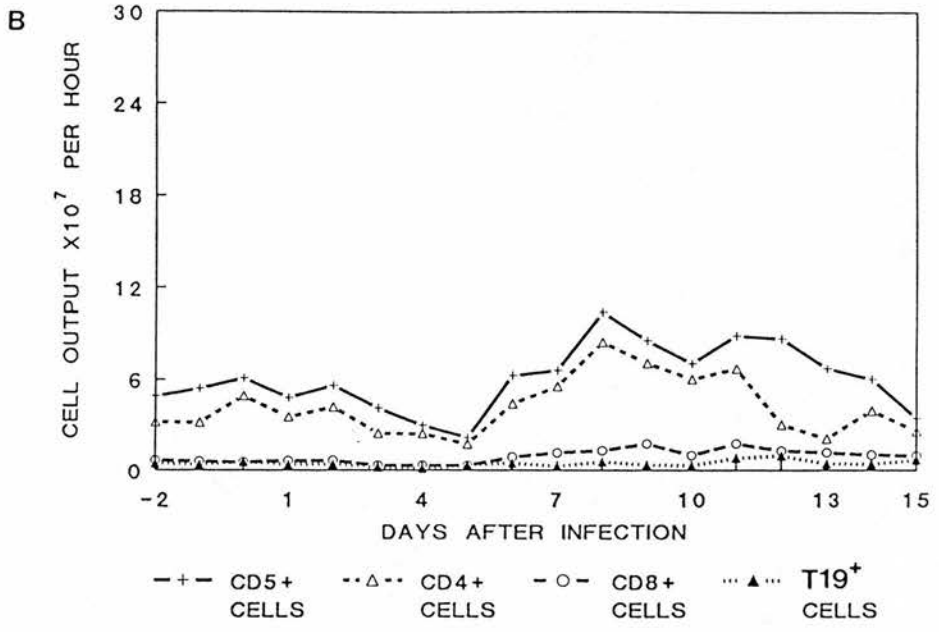
TRYPS./ML (LOG 10)
DAYS AFTER INFECTION
CELL OUTPUT
BLAST CELLS
TRYPS. /ML


B
CELL OUTPUT X10⁷ PER HOUR
DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS
T19+ CELLS

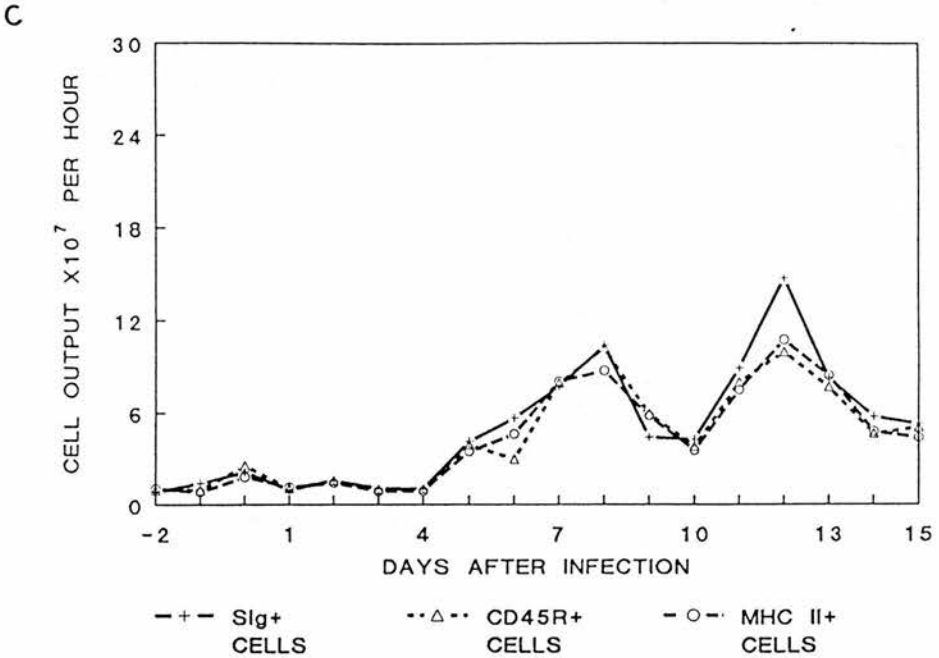

C
CELL OUTPUT X10⁷ PER HOUR
DAYS AFTER INFECTION
SIg+ CELLS
CD45R+ CELLS
MHC II+ CELLS

TABLE E 4.4 Lymph flow, cellular responses and parasite kinetics in prefemoral efferent lymph of sheep 749 after infection in drainage area with *T. congolense* TREU 1457

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^7$)	Parasitosis (log_{10} tryps/ml)	Lymphoblast output $x10^7$/hr (%)
-5	75 (18 hrs)	ND	-	-	ND -
-4	150	ND	-	-	ND -
-3	199	ND	-	-	0.2 (2)
-2	255	9.9	10.5	-	0.2 (2)
-1	280	6.5	7.6	-	0.2 (3)
0	255	7.4	7.9	-	0.04 (0.5)
1	210	6.7	5.9	-	0 (0)
2	188	8.5	6.7	-	0.3 (5)
3	150	14.1	8.8	3.8	0.4 (4.5)
4	132	7.3	4.0	4.7	0.6 (14.5)
5	128	10.8	5.8	5.6	1.1 (19.5)
6	110	21.4	9.8	5.3	2.5 (25.5)
7	126	29.4	15.4	5.6	3.98 (25.5)
8	130	40.0	21.7	5.9	4.97 (23.0)
9	120	27.9	14.0	6.2	2.80 (20.0)
10	124	22.5	11.6	6.5	1.11 (9.5)
11	120	34.9	17.5	6.1	3.85 (22.0)
12	134	33.7	18.8	5.8	4.35 (23.0)
13	100	32.1	13.4	5.9	2.30 (17.0)
14	106	26.7	11.8	5.9	0.88 (7.5)
15	57 (12 hrs)	18.5	8.8	5.3	0.59 (8.0)

TABLE E 4.5 T lymphocyte subpopulation dynamics in prefemoral efferent lymph of sheep 749 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells		$CD4^+$ cells		$CD8^+$ cells		SBU-$T19^+$ cells	
-2	4.9	(83.4)	3.18	(73.3)	0.64	(6.1)	0.42	(4.0)
-1	5.4	(71.3)	3.16	(40.7)	0.58	(7.6)	0.35	(4.6)
0	6.1	(76.5)	4.91	(61.7)	0.53	(6.7)	0.53	(6.7)
1	4.8	(80.2)	3.52	(60.0)	0.62	(10.5)	0.35	(5.9)
2	5.6	(83.8)	4.18	(62.7)	0.63	(9.4)	0.39	(5.8)
3	4.1	(46.6)	2.43	(27.6)	0.31	(3.5)	0.19	(2.2)
4	2.96	(73.8)	2.41	(60.5)	0.33	(8.4)	0.11	(2.8)
5	2.15	(36.3)	1.72	(39.9)	0.32	(6.0)	0.32	(7.4)
6	6.27	(69.1)	4.40	(46.3)	0.87	(10.6)	0.46	(3.0)
7	6.60	(51.2)	5.55	(40.8)	1.15	(12.3)	0.26	(9.7)
8	10.42	(59.8)	8.46	(46.9)	1.30	(12.3)	0.54	(10.6)
9	8.58	(66.8)	7.08	(49.1)	1.76	(9.2)	0.35	(5.1)
10	7.06	(68.7)	6.03	(54.5)	0.98	(6.2)	0.31	(2.6)
11	8.89	(55.8)	6.72	(40.6)	1.76	(14.3)	0.80	(11.7)
12	8.70	(50.0)	3.01	(36.7)	1.28	(5.2)	0.98	(6.5)
13	6.72	(42.1)	2.09	(31.3)	1.17	(3.3)	0.95	(3.8)
14	6.01	(43.3)	3.93	(37.1)	1.02	(2.6)	0.42	(4.3)
15	3.42	(38.0)	2.56	(29.3)	0.95	(1.8)	0.67	(4.5)

TABLE E 4.6 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in prefemoral efferent lymph of sheep 749 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	MHC Class II$^+$ (SW73.2) cells	MHC Class II$^+$ (SBU-II) cells	CD45R$^+$ cells	SIg$^+$ cells
-2	1.06 (9.7)	1.06 (9.7)	0.95 (9.0)	0.85 (8.0)
-1	1.29 (16.5)	0.82 (11.0)	0.94 (12.7)	1.40 (18.7)
0	1.60 (19.7)	1.81 (23.1)	2.56 (33.1)	2.14 (26.8)
1	0.79 (13.4)	1.14 (19.7)	1.06 (17.4)	0.97 (15.7)
2	1.02 (15.1)	1.42 (21.2)	1.58 (23.1)	1.58 (23.1)
3	3.91 (44.4)	0.87 (9.9)	1.00 (11.4)	1.06 (12.0)
4	1.15 (28.8)	0.88 (21.4)	1.04 (26.3)	1.04 (26.3)
5	2.74 (47.0)	3.49 (60.2)	3.92 (68.0)	4.14 (71.1)
6	3.98 (40.6)	4.63 (47.1)	2.98 (30.4)	5.68 (58.0)
7	5.29 (34.3)	8.07 (52.3)	7.96 (51.8)	7.75 (50.4)
8	5.64 (25.9)	8.79 (40.6)	10.25 (47.2)	10.42 (48.0)
9	5.17 (37.0)	5.82 (41.4)	5.97 (42.8)	4.42 (31.6)
10	3.87 (33.4)	3.56 (30.8)	3.82 (32.7)	4.28 (36.7)
11	8.52 (48.8)	7.52 (43.0)	8.02 (45.9)	8.93 (50.9)
12	10.54 (56.1)	10.77 (57.3)	9.99 (53.1)	14.73 (78.4)
13	8.10 (60.4)	8.43 (63.0)	7.68 (57.4)	8.31 (61.9)
14	6.05 (51.3)	4.77 (40.5)	4.64 (39.2)	5.75 (48.8)
15	4.14 (47.2)	4.38 (49.9)	5.04 (57.5)	5.28 (60.1)

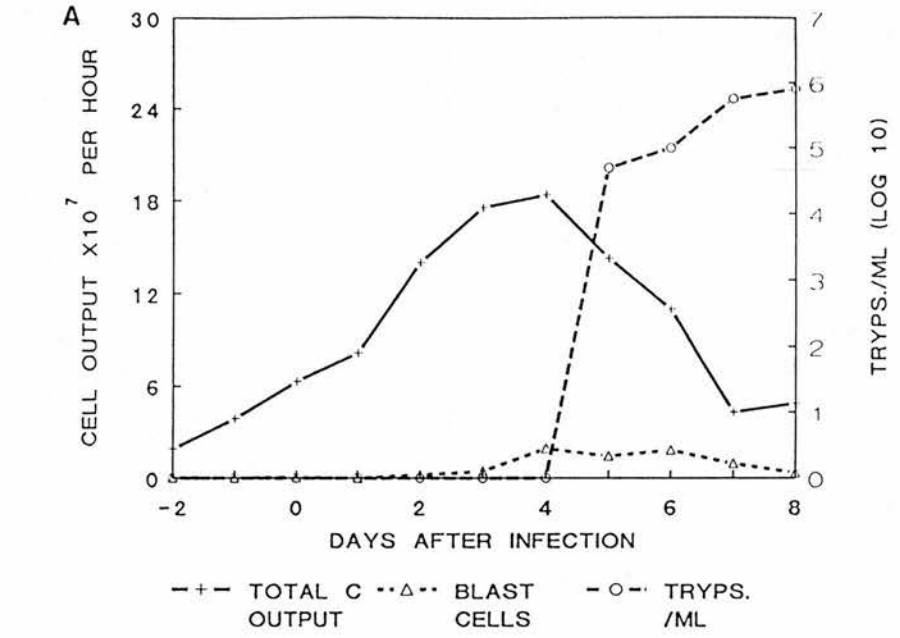
FIGURE E 4.2 Cellular phenotype dynamics and parasite kinetics on prefemoral efferent lymph from lymph nodes draining local skin reactions in sheep 770 infected with *T. congolense* TREU 1457

(A) Parasite kinetics
Total cellular output
Lymphoblast output

(B) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

(C) SIg^+ cells
$CD45R^+$ cells
MHC Class II^+ cells

E4.2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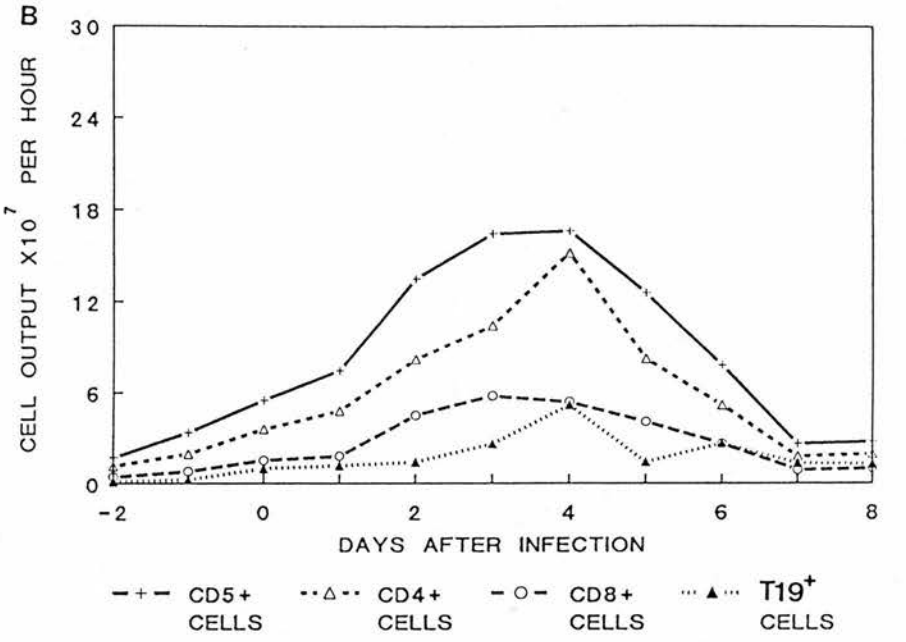
TRYPS./ML (LOG 10)
DAYS AFTER INFECTION
TOTAL C OUTPUT
BLAST CELLS
TRYPS. /ML


B
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DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS
T19+ CELLS

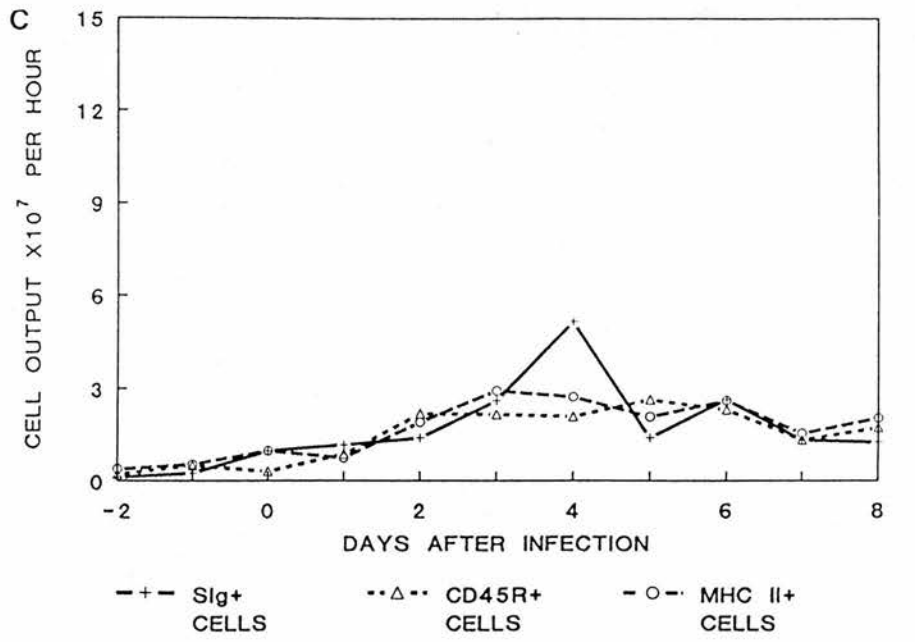

C
CELL OUTPUT X10⁷ PER HOUR
DAYS AFTER INFECTION
SIg+ CELLS
CD45R+ CELLS
MHC II+ CELLS

TABLE E 4.7 **Lymph flow, cellular responses and parasite kinetics in prefemoral efferent of sheep 770 after infection in drainage area with *T. congolense* TREU 1457**

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^7$)	Parasitosis (log_{10} tryps/ml)	Lymphoblasts $x10^7$/hr (%)
-5	85 (18 hrs)	-	-	-	- -
-4	35 (?)	-	-	-	- -
-3	115	1.15	-	-	0.02 (1.5)
-2	152	1.90	-	-	0.02 (1.0)
-1	206	4.5	3.86	-	0.02 (0.5)
0	172	8.8	6.31	-	0.03 (0.5)
1	178	11.0	8.16	-	0 0
2	190	17.7	14.0	-	0.21 (1.5)
3	172	24.5	17.56	-	0.44 (2.5)
4	170	25.9	18.35	-	1.93 (10.5)
5	168	20.4	14.28	5.7	1.43 (10.0)
6	190	13.9	11.00	6.0	1.82 (16.5)
7	111	9.3	4.30	6.75	0.95 (22.0)
8	110	10.6	4.86	6.90	0.34 (7.0)

TABLE E 4.8 **T lymphocyte subpopulation dynamics in prefemoral efferent lymph of sheep 770 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)**

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
-2	1.70 (89.3)	1.13 (59.3)	0.42 (21.9)	0.11 (6.0)
-1	3.36 (87.1)	1.94 (50.2)	0.78 (20.3)	0.24 (6.1)
0	5.50 (87.2)	3.59 (56.9)	1.52 (24.1)	0.38 (6.0)
1	7.45 (91.3)	4.77 (58.5)	1.80 (22.1)	1.47 (18.0)
2	13.44 (96.0)	8.19 (58.5)	4.49 (32.1)	0.74 (5.3)
3	16.40 (93.4)	10.41 (59.3)	5.78 (32.9)	0.86 (4.9)
4	16.59 (90.4)	15.16 (82.6)	5.38 (29.3)	1.38 (7.5)
5	12.55 (87.9)	8.24 (57.7)	4.07 (28.5)	0.54 (3.8)
6	7.81 (71.0)	5.17 (47.0)	2.63 (23.9)	0.40 (3.6)
7	2.61 (60.8)	1.78 (41.3)	0.89 (20.6)	0.15 (3.5)
8	2.75 (56.7)	1.93 (39.8)	0.99 (20.3)	0.22 (4.6)

TABLE E 4.9 **Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in prefemoral efferent of sheep 770 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)**

Days after infection	MHC Class II$^+$ (SW73.2) cells	MHC Class II$^+$ (SBU-II) cells	CD45R$^+$ cells	SIg$^+$ cells
-2	0.25 (13.3)	0.38 (19.8)	0.21 (10.9)	0.12 (6.1)
-1	0.45 (11.7)	0.53 (13.7)	0.49 (12.7)	0.24 (6.2)
0	0.65 (10.3)	0.97 (15.4)	0.30 (4.8)	1.97 (15.3)
1	2.24 (27.5)	0.74 (9.1)	0.86 (10.5)	1.16 (14.2)
2	3.71 (26.5)	1.90 (13.6)	2.18 (15.6)	1.39 (9.9)
3	3.94 (22.5)	2.92 (16.7)	2.15 (12.3)	2.60 (14.8)
4	5.62 (30.6)	2.73 (14.9)	2.09 (11.4)	5.16 (28.1)
5	2.18 (15.3)	2.08 (14.6)	2.64 (18.5)	1.40 (9.8)
6	2.64 (24.0)	2.61 (23.7)	2.31 (21.0)	2.64 (15.0)
7	1.32 (30.8)	1.54 (35.8)	1.32 (30.7)	1.32 (22.3)
8	1.55 (32.0)	2.04 (42.1)	1.73 (35.5)	1.26 (25.9)

FIGURE E 4.3 Cellular phenotype dynamics and parasite kinetics in prefemoral efferent lymph from lymph nodes draining local skin reactions in sheep 742 infected with *T. congolense* TREU 1457

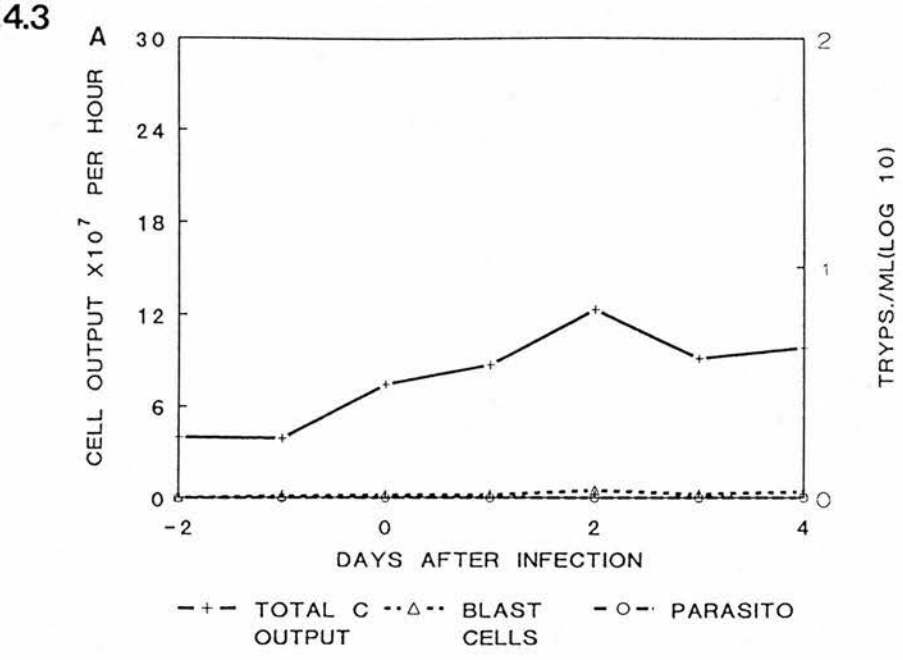
(A) Parasite kinetics
Total cellular output
Lymphoblast output

(B) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

(C) SIg^+ cells
$CD45R^+$ cells
MHC Class II^+ cells

E 4.3

0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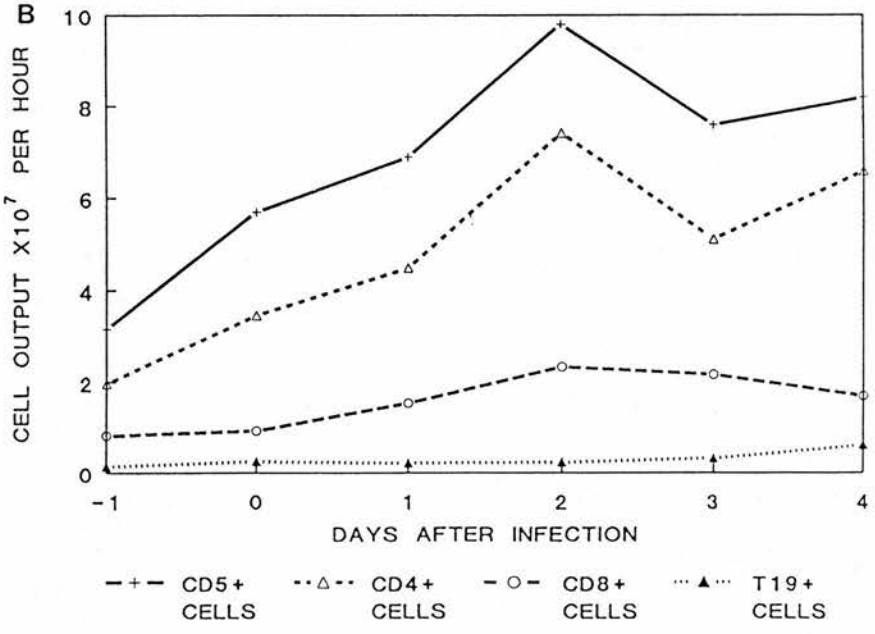
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
TOTAL C OUTPUT
BLAST CELLS
PARASITO


B
CELL OUTPUT X10⁷ PER HOUR
DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS
T19+ CELLS

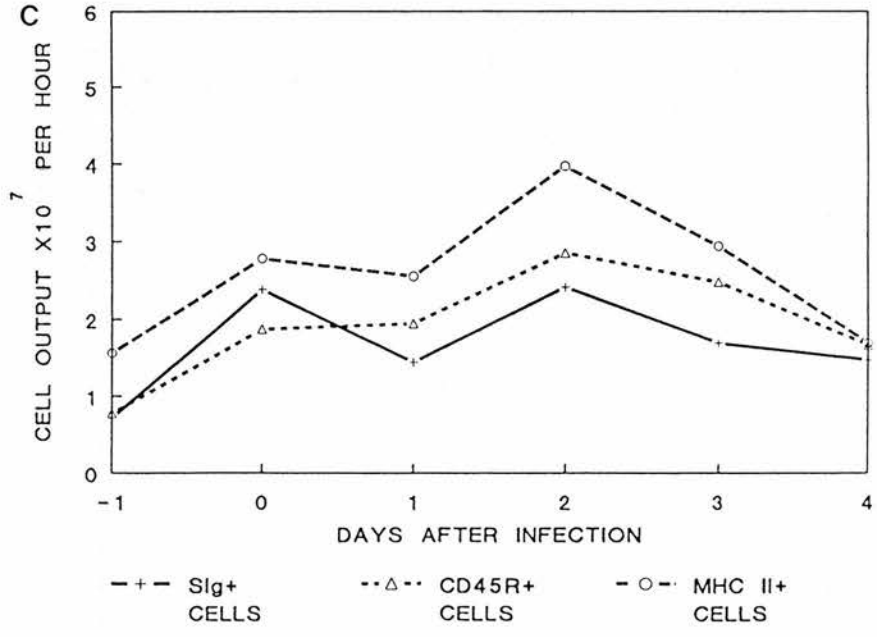

C
CELL OUTPUT X10⁷ PER HOUR
DAYS AFTER INFECTION
SIg+ CELLS
CD45R+ CELLS
MHC II+ CELLS

TABLE E 4.10 **Lymph flow, cellular responses and parasite kinetics in prefemoral efferent lymph of sheep 742 after infection in drainage area with *T. congolense* TREU 1457**

Days after infection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^7$)	Parasitosis (log_{10} tryps)	Lymphoblasts $x10^7$hr(%)
-2	62 (20 hrs)	ND	ND	-	ND-
-1	140	6.7	3.9	-	0.04 (1)
0	130	13.7	7.4	-	0.15 (2)
1	121	17.3	8.7	-	0.26 (3)
2	150	19.8	12.3	-	0.17 (2)
3	124	17.7	9.1	-	0.18 (1.5)
4	175	13.4	9.8	-	0.36 (4.0)
5	-				

TABLE E 4.11 T lymphocyte subpopulation dynamics in prefemoral efferent lymph of sheep 742 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
-2	ND-	-	-	-
-1	3.16 (81.0)	1.96 (50.1)	0.82 (21.1)	0.14 (3.7)
0	5.7 (76.9)	3.46 (46.7)	0.93 (12.5)	0.25 (3.4)
1	6.9 (79.8)	4.49 (51.7)	1.54 (17.7)	0.22 (2.5)
2	9.8 (79.9)	7.42 (60.3)	2.33 (18.9)	0.23 (2.6)
3	7.6 (83.9)	5.11 (56.2)	2.16 (23.7)	0.32 (3.5)
4	8.2 (83.5)	6.59 (67.3)	1.69 (17.2)	0.61 (6.2)

TABLE E 4.12 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in prefermoral efferent lymph of sheep 742 infected with *T. congolense* TREU 1457. Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	MHC Class II$^+$ (SW73.2) cells	MHC Class II$^+$ (SBU-11) cells	CD45R$^+$ cells	SIg$^+$ cells
-2	ND-	ND-	ND-	ND-
-1	1.25 (32.1)	1.56 (40.1)	0.78 (19.9)	0.72 (24.1)
0	1.80 (24.3)	2.78 (37.7)	1.87 (25.3)	2.38 (32.2)
1	1.73 (19.9)	2.55 (29.3)	1.94 (22.3)	1.44 (16.5)
2	2.96 (24.1)	3.97 (32.3)	2.85 (23.2)	2.41 (19.6)
3	2.78 (30.6)	2.94 (33.3)	2.48 (27.3)	1.69 (18.6)
4	1.74 (17.8)	1.69 (17.2)	1.66 (16.9)	1.47 (15.0)

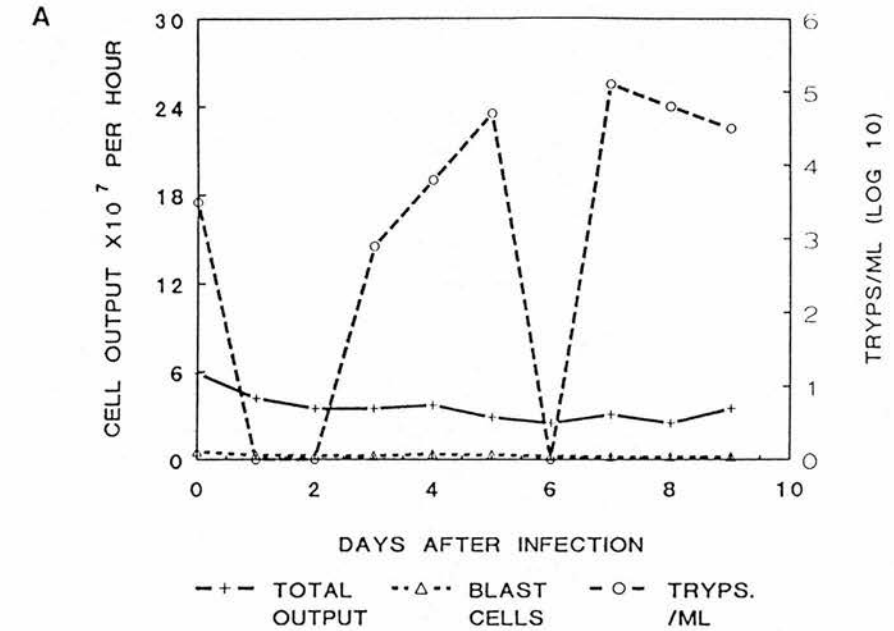
FIGURE E 4.4 Cellular phenotype dynamics and parasite kinetics in prefemoral efferent lymph from lymph nodes draining local skin reactions in sheep 758 superinfected with *T. congolense* TREU 1881

(A) Parasite kinetics
Total cellular output
Lymphoblast output

(B) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells
SBU-$T19^+$ cells

(C) SIg^+ cells
$CD45R^+$ cells
MHC Class II^+ cells

E4.4


A
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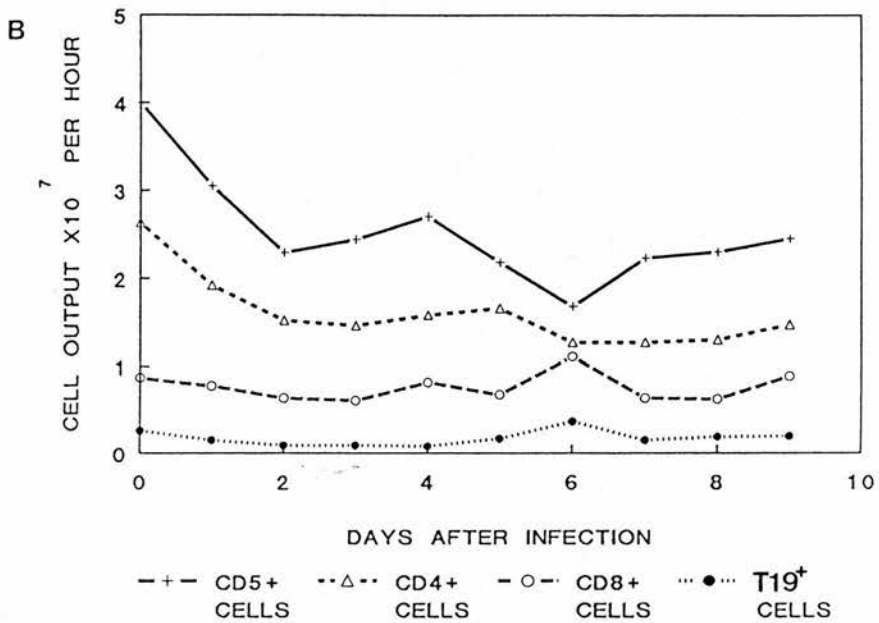
TRYPS/ML (LOG 10)
DAYS AFTER INFECTION
TOTAL OUTPUT
BLAST CELLS
TRYPS. /ML


B
CELL OUTPUT X10 7 PER HOUR
DAYS AFTER INFECTION
CD5+ CELLS
CD4+ CELLS
CD8+ CELLS
T19+ CELLS

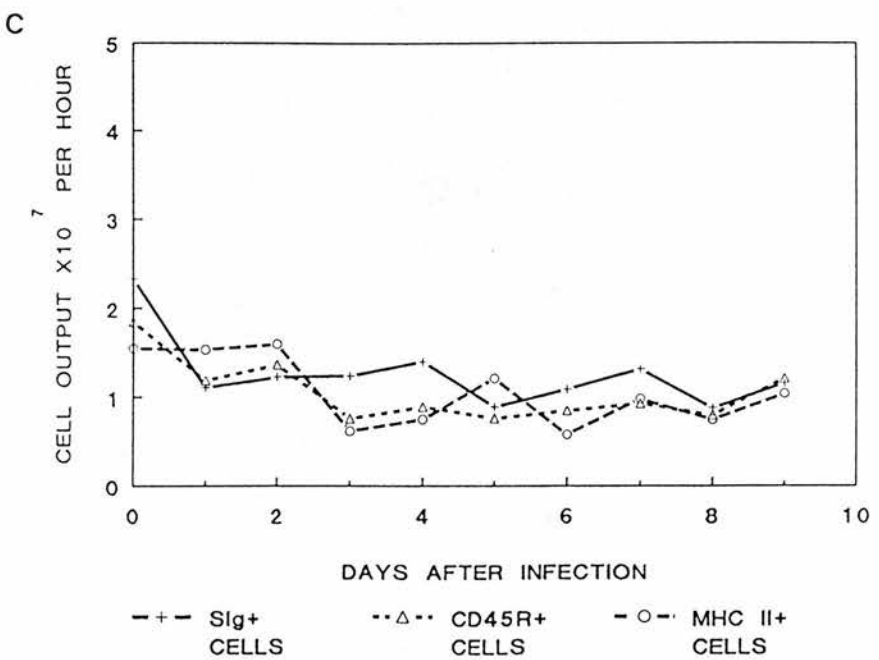

C
CELL OUTPUT X10 7 PER HOUR
DAYS AFTER INFECTION
SIg+ CELLS
CD45R+ CELLS
MHC II+ CELLS

TABLE E 4.13 Lymph flow, cellular responses and parasite kinetics in prefemoral efferent lymph of sheep 758 after superinfection in drainage area with an heterologous *T. congolense* (TREU 1881)

Days after superinfection	Lymph flow (mls per day)	Cell content ($x10^6$/ml)	Hourly cell output($x10^7$)	Parasitosis (log_{10} tryps/ml)	Lymphocytes $x10^7$/hr(%)
0	100	14.2	5.9	3.5	0.53 (9.0)
1	116	8.6	4.2	0	0.29 (7.0)
2	150	5.6	3.5	0	0.28 (8.0)
3	80	10.5	3.5	2.9	0.25 (7.0)
4	89	10.1	3.7	3.8	0.37 (10.0)
5	77	8.9	2.9	4.7	0.32 (11.0)
6	92	6.5	2.5	0	0.23 (9.0)
7	105	7.0	3.1	5.1	0.19 (6.0)
8	89	6.8	2.5	4.8	0.19 (7.5)
9	133	6.3	3.5	4.5	0.18 (5.0)

TABLE E 4.14 T lymphocyte subpopulation dynamics in prefemoral efferent lymph of sheep 758 after superinfection with heterologous *T. congolense* (TREU 1881). Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	$CD5^+$ cells	$CD4^+$ cells	$CD8^+$ cells	SBU-$T19^+$ cells
0	3.94 (66.7)	2.63 (44.5)	0.87 (14.8)	0.26 (4.4)
1	3.05 (72.7)	1.92 (45.8)	0.78 (18.6)	0.15 (3.6)
2	2.29 (65.5)	1.52 (43.3)	0.64 (18.4)	0.09 (2.8)
3	2.44 (69.6)	1.46 (41.8)	0.61 (17.4)	0.09 (2.6)
4	2.7 (73.0)	1.58 (42.7)	0.82 (22.1)	0.08 (2.1)
5	2.18 (75.2)	1.66 (57.4)	0.68 (23.5)	0.17 (5.9)
6	1.68 (67.3)	1.27 (50.8)	1.11 (44.5)	0.37 (14.9)
7	2.23 (72.0)	1.27 (41.0)	0.64 (20.6)	0.15 (4.7)
8	2.3 (92.0)	1.3 (52.0)	0.63 (25.2)	0.19 (7.6)
9	2.45 (70.0)	1.47 (42.0)	0.89 (25.4)	0.2 (5.7)

TABLE E. 4.15 Dynamics of CD1$^+$, MHC Class II$^+$, CD45R$^+$ and SIg$^+$ cells in prefemoral efferent lymph of sheep 758 superinfected with heterologous *T. congolense* (TREU 1881). Absolute cell output x10^7/hr and relative proportions (percent fluorescent cells)

Days after infection	MHC Class II$^+$ (SW73.2) cells	MHC Class II$^+$ SBU-11 cells	CD45R$^+$ cells	SIg$^+$ cells
0	1.68 (28.4)	1.55 (26.2)	1.85 (31.4)	2.33 (39.6)
1	1.18 (28.1)	1.54 (36.7)	1.19 (28.4)	1.11 (26.4)
2	1.84 (52.5)	1.60 (45.7)	1.37 (39.1)	1.23 (35.2)
3	1.15 (32.9)	0.62 (17.8)	0.76 (21.8)	1.24 (35.5)
4	1.07 (28.8)	0.75 (20.2)	0.89 (24.0)	1.4 (38.0)
5	0.61 (21.1)	1.20 (41.7)	0.76 (26.1)	0.89 (30.7)
6	0.54 (21.6)	0.58 (23.1)	0.85 (34.1)	1.09 (43.5)
7	0.98 (31.7)	0.86 (27.9)	0.93 (32.0)	1.32 (37.8)
8	0.90 (36.0)	0.75 (30.0)	0.8 (32.0)	0.88 (35.2)
9	0.99 (28.3)	1.04 (34.7)	1.21 (34.6)	1.16 (33.1)

APPENDIX V
PERIPHERAL BLOOD DATA

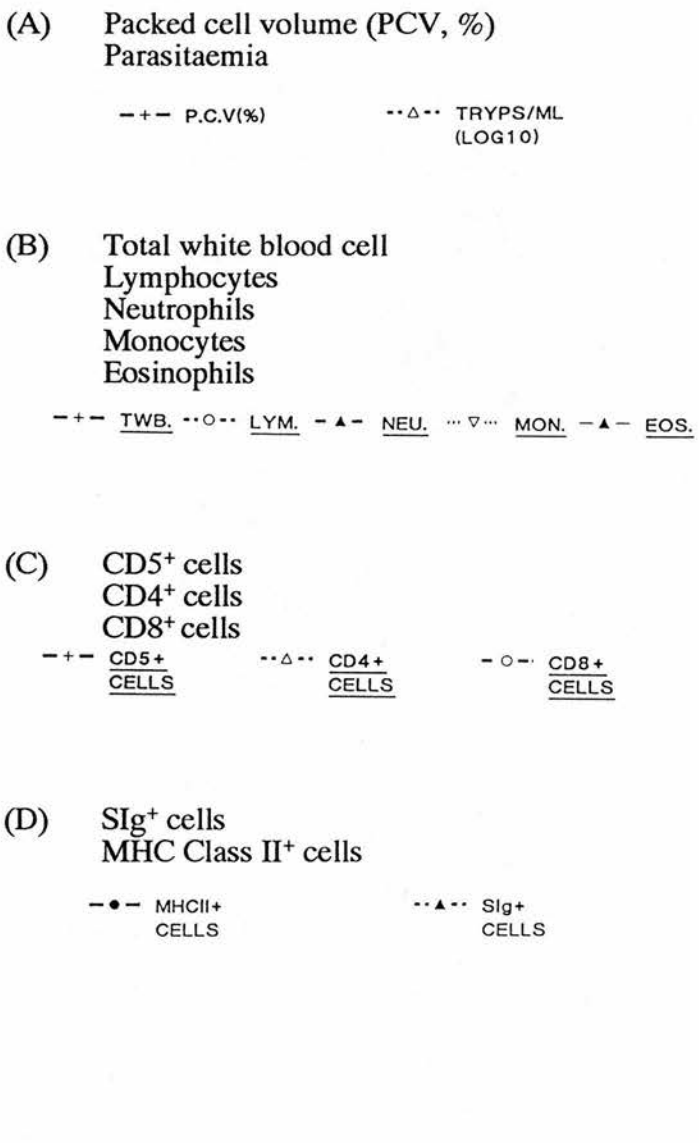
TABLE P 5.1 Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 101 infected with *T. congolense* TREU 1457

Days after infection	PCV(%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia Tryps/ml (log_{10})
-2	37	8.1	5.4	-
-1	38.0	7.7	5.4	-
5	38.0	8.0	6.0	-
8	37.0	6.8	4.8	-
12	35.0	6.3	5.0	-
15	37.0	7.4	5.6	-
19	35.0	8.2	6.6	2
22	33.0	8.6	6.7	6.6
25	32.0	13.0	11.1	6.6
29	30.0	12.3	10.6	6.9
32	28.0	12.8	10.8	6.6
36	29.0	11.8	11.3	6.3
41	29.0	12.7	11.3	6.9
44	27.0	10.9	7.8	6.6
47	26.0	12.4	10.7	6.3
50	31.0	13.7	11.9	6.3
54	27.0	13.0	11.4	6.9
57	27.0	13.2	12.0	6.6
61	-	-	-	-
Trypanocidal drug therapy				
64	25.0	11.5	10.8	-
69	29.0	9.0	8.1	-
72	28.0	7.5	6.5	-
76	31.0	8.2	6.2	-
79	32.0	10.7	8.5	-

TABLE P 5.2 Peripheral blood leucocyte subpopulation dynamics in sheep 101 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-2	1.6 (30.0)	1.3 (25.0)	0.7 (13)	1.6 (29.0)	1.4 (25.0)
-1	1.7 (31.2)	1.3 (24.5)	0.7 (12.6)	1.6 (29.0)	1.4 (25.8)
5	2.1 (35.1)	0.9 (15.7)	0.7 (11.3)	1.4 (23.7)	1.3 (22.3)
8	1.9 (39.0)	1.0 (20.7)	1.1 (22.8)	1.3 (26.7)	1.5 (31.6)
12	1.4 (27.4)	0.7 (13.5)	0.7 (14.8)	1.5 (30.7)	1.9 (37.6)
15	1.4 (25.1)	0.7 (11.9)	0.4 (6.8)	1.0 (16.9)	0.7 (17.3)
19	1.9 (28.8)	1.1 (16.1)	1.0 (14.9)	1.9 (28.9)	2.7 (40.2)
22	- -	- -	- -	- -	- -
25	2.6 (23.6)	1.2 (11.2)	0.6 (5.3)	5.0 (45.0)	6.4 (57.9)
29	2.1 (19.8)	1.7 (16.4)	0.8 (7.3)	3.4 (32.2)	5.9 (55.6)
32	4.6 (43.2)	1.4 (12.7)	0.6 (5.3)	7.0 (64.8)	6.1 (56.7)
36	3.6 (31.5)	1.1 (9.8)	0.6 (5.6)	2.0 (17.3)	4.4 (38.8)
41	2.2 (19.6)	1.5 (13.1)	0.9 (8.0)	4.3 (37.8)	6.1 (53.6)
44	1.9 (24.1)	1.2 (15.8)	0.5 (6.6)	2.8 (35.6)	4.2 (53.6)
47	2.4 (22.8)	1.6 (24.8)	1.2 (10.8)	3.4 (31.8)	4.7 (43.8)
50	1.7 (14.2)	0.8 (7.0)	0.9 (7.4)	8.1 (68.9)	7.3 (61.1)
54	1.6 (14.4)	0.7 (6.2)	0.6 (5.1)	7.7 (67.2)	5.5 (48.1)
57	1.9 (15.4)	1.0 (8.3)	0.6 (5.3)	3.3 (27.1)	3.9 (32.8)
61	- -	- -	- -	- -	- -
Trypanocidal drug therapy					
64	2.7 (25.2)	1.7 (16.1)	0.6 (5.3)	7.8 (72.4)	5.9 (54.7)
69	2.9 (35.3)	1.9 (23.6)	1.2 (13.5)	2.7 (32.8)	4.0 (49.1)
72	1.8 (27.4)	1.1 (16.3)	0.8 (12.6)	3.6 (56.2)	3.0 (46.3)
76	1.6 (25.5)	0.8 (12.3)	0.6 (9.4)	3.2 (52.6)	2.3 (37.4)
79	2.8 (33.7)	1.4 (16.8)	1.2 (14.1)	4.4 (52.0)	2.4 (40.5)

FIGURE P 5.1 Peripheral blood leucocyte subpopulation dynamics, parasitaemia and changes in PCV in sheep 103 infected with *T. congolense* TREU 1457



P5.1

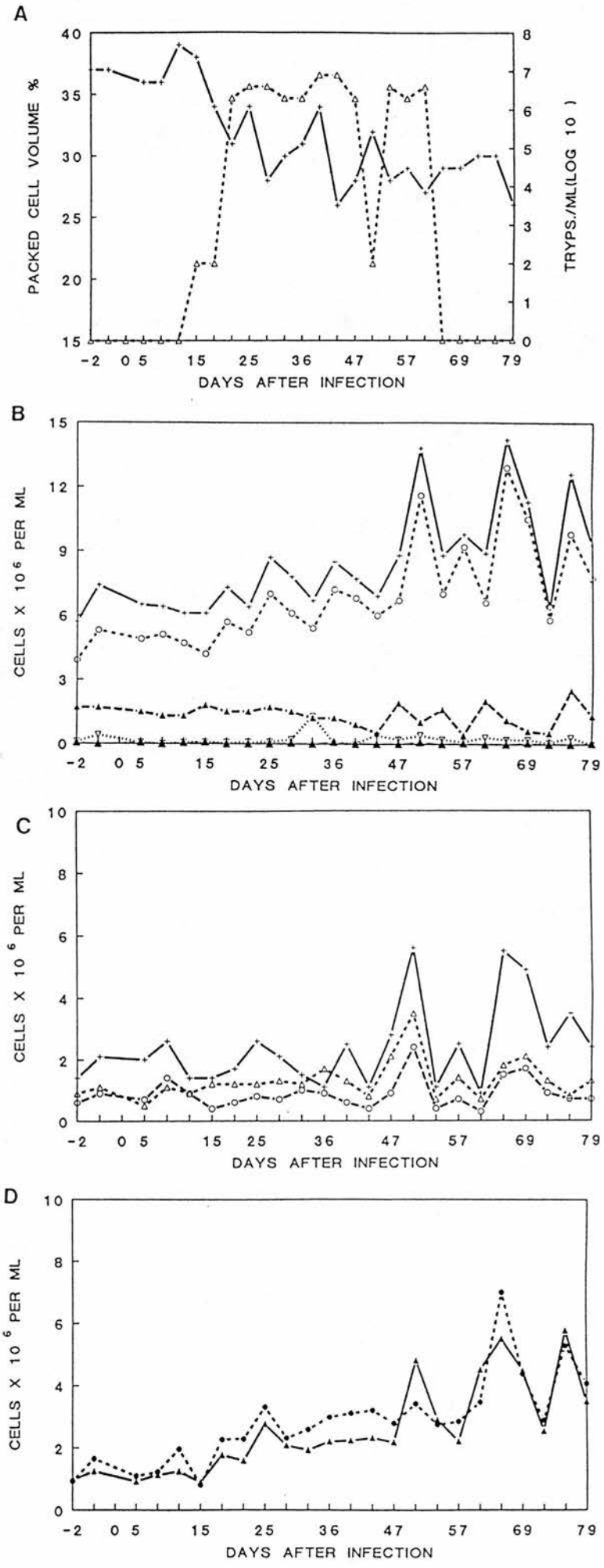

A
PACKED CELL VOLUME %
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
B
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
C
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
D
CELLS X 10 6 PER ML
DAYS AFTER INFECTION

TABLE P 5.3 **Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 103 infected with *T. congolense* TREU 1457**

Days after infection	PCV(%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia tryps/ml (log_{10})
-2	37.0	5.7	3.9	-
-1	37.0	7.4	5.3	-
5	36.0	6.5	4.9	-
8	36.0	6.4	5.1	-
12	39.0	6.1	4.7	-
15	38.0	6.1	4.2	2.0
19	34.0	7.3	5.7	2.0
22	31.0	6.4	5.2	6.3
25	34.0	8.7	6.9	6.6
29	28.0	7.8	6.1	6.6
32	30.0	6.7	5.4	6.3
36	31.0	8.5	7.2	6.3
41	34.0	7.7	6.8	6.9
44	26.0	6.9	6.0	6.9
47	28.0	8.8	6.7	6.3
50	32.0	13.8	11.6	2.0
54	28.0	8.8	7.0	6.6
57	29.0	9.8	9.2	6.3
61	27.0	8.9	6.6	6.6
Trypanocidal drug therapy				
64	29.0	14.2	12.9	-
69	29.0	11.3	10.5	-
72	30.0	6.3	5.8	-
76	30.0	12.6	9.8	-
79	26.0	9.3	7.7	-

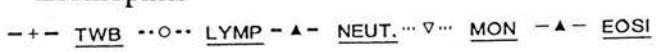
TABLE P 5.4 **Peripheral blood leucocyte subpopulation dynamics in sheep 103 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)**

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-2	1.4 (37.0)	0.9 (24.0)	0.6 (14.0)	1.0 (25.0)	0.9 (24.0)
-1	2.1 (39.8)	1.1 (38.8)	0.9 (16.7)	1.3 (23.5)	1.7 (30.9)
5	2.0 (41.2)	0.5 (10.7)	0.7 (14.1)	0.9 (18.4)	1.1 (22.4)
8	2.6 (51.5)	1.1 (22.3)	1.4 (28.1)	1.1 (22.0)	1.2 (23.9)
12	1.4 (28.6)	0.9 (19.1)	0.9 (19.1)	1.2 (26.2)	2.0 (41.3)
15	1.4 (33.0)	1.2 (29.1)	0.4 (8.5)	0.9 (21.3)	0.8 (19.4)
19	1.7 (29.5)	1.2 (21.5)	0.6 (10.8)	1.8 (30.7)	2.3 (39.4)
22	- -	- -	- -	- -	- -
25	2.6 (37.1)	1.2 (17.5)	0.8 (11.6)	2.8 (39.6)	3.3 (47.5)
29	2.1 (25.3)	1.3 (21.8)	0.7 (12.2)	2.1 (34.2)	2.3 (38.0)
32	1.5 (26.9)	1.2 (22.6)	1.0 (18.9)	1.9 (35.7)	2.6 (48.1)
36	1.1 (17.1)	1.7 (24.0)	0.9 (12.7)	2.2 (30.7)	3.0 (41.6)
41	2.5 (37.5)	1.3 (19.0)	0.6 (8.9)	2.2 (33.1)	3.1 (46.1)
44	1.1 (20.3)	0.8 (22.5)	0.4 (7.5)	2.3 (38.7)	3.2 (53.5)
47	2.8 (42.1)	2.1 (31.6)	0.9 (14.4)	2.2 (32.6)	2.8 (41.7)
50	5.6 (48.5)	3.5 (29.8)	2.4 (21.1)	4.8 (41.5)	3.4 (29.5)
54	1.1 (15.9)	0.7 (15.9)	0.4 (9.3)	2.9 (41.5)	2.8 (39.1)
57	2.5 (27.7)	1.4 (14.8)	0.7 (11.6)	2.2 (24.1)	2.9 (31.1)
61	0.9 (13.2)	0.7 (10.8)	0.3 (9.3)	4.5 (68.8)	3.5 (53.1)
Trypanocidal drug therapy					
64	5.5 (43.0)	1.8 (14.1)	1.4 (12.0)	5.5 (42.9)	7.0 (54.5)
69	4.9 (46.6)	2.1 (19.8)	1.7 (16.0)	4.5 (42.9)	4.4 (41.9)
72	2.4 (42.1)	1.3 (22.2)	0.9 (14.9)	2.5 (44.1)	2.9 (50.3)
76	3.5 (35.6)	0.8 (8.5)	0.7 (6.9)	5.8 (58.9)	5.3 (53.8)
79	2.4 (31.0)	1.3 (17.2)	0.7 (9.5)	3.4 (45.4)	4.1 (52.9)

FIGURE P 5.2 Peripheral blood leucocyte subpopulation dynamics, parasitaemia and changes in PCV in sheep 104 infected with *T. congolense* TREU 1457

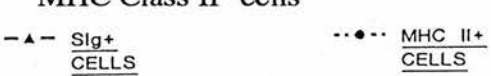
(A) Packed cell volume (PCV, %)
Parasitaemia

(B) Total white blood cell
Lymphocytes
Neutrophils
Monocytes
Eosinophils



(C) $CD5^+$ cells
$CD4^+$ cells
$CD8^+$ cells

(D) SIg^+ cells
MHC Class II^+ cells



P5.2

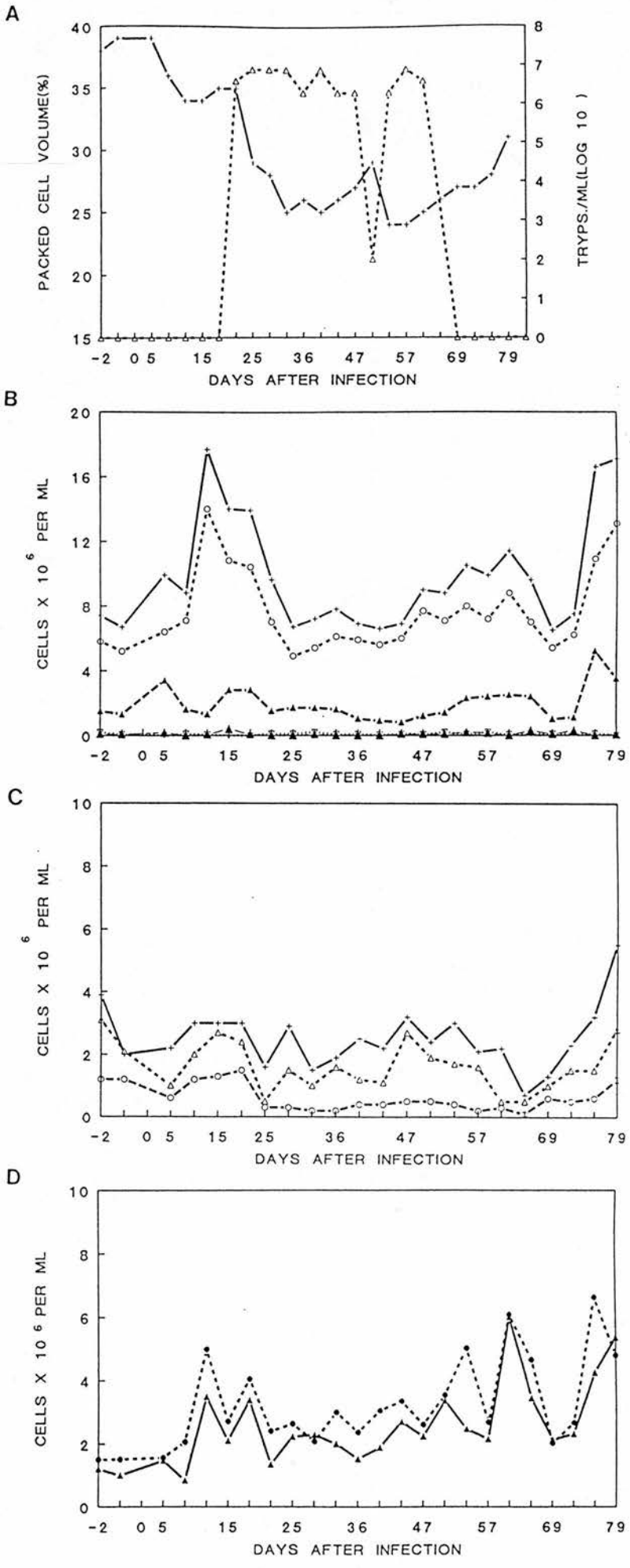

A
PACKED CELL VOLUME(%)
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
B
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
C
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
D
CELLS X 10 6 PER ML
DAYS AFTER INFECTION

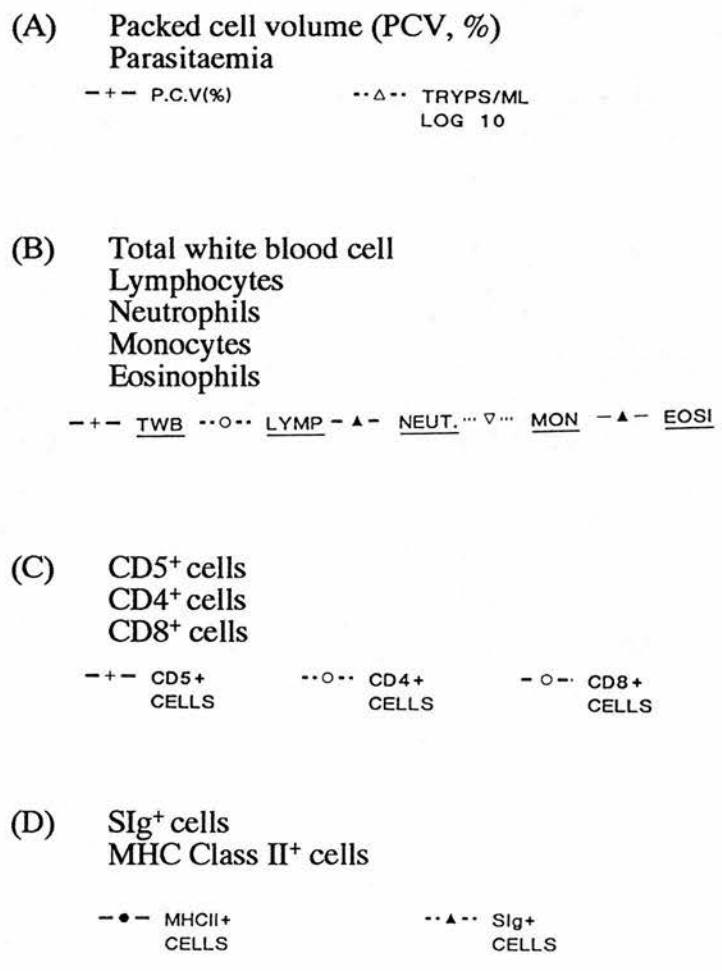
TABLE P 5.5 **Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 104 infected with *T. congolense* TREU 1457**

Days after infection	PCV (%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia tryps/ml (log_{10})
-2	38.0	7.4		-
-1	39.0	6.7	5.2	-
5	39.0	9.9	6.4	-
8	36.0	8.8	7.1	-
12	34.0	17.7	14.0	-
15	34.0	14.0	10.9	-
19	35.0	13.9	10.4	-
22	35.0	9.6	7.0	6.6
25	29.0	6.7	4.9	6.9
29	28.0	7.2	5.4	6.9
32	25.0	7.8	6.1	6.9
36	26.0	6.9	5.9	6.3
41	25.0	6.6	5.6	6.9
44	26.0	6.9	6.0	6.3
47	27.0	9.0	7.7	6.3
50	29.0	8.8	7.1	2.0
54	24.0	10.5	8.0	6.3
57	24.0	9.9	7.2	6.9
61	25.0	14.0	8.8	6.6
Trypanocidal drug therapy				
64	26.0	9.6	7.0	-
69	27.0	6.5	5.4	-
72	27.0	7.5	6.2	-
76	28.0	16.6	10.9	-
79	31.0	17.1	13.1	

TABLE P 5.6 Peripheral blood leucocyte subpopulation dynamics in sheep 104 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-2	3.9 (38.0)	3.1 (30.0)	1.2 (12.0)	1.2 (27.0)	1.5 (2.9)
-1	2.0 (39.0)	2.1 (41.9)	1.2 (12.0)	1.0 (19.1)	1.5 (28.8)
5	2.2 (33.6)	1.0 (16.3)	0.6 (11.2)	1.5 (22.8)	2.1 (29.1)
8	3.0 (42.9)	2.0 (28.2)	1.2 (19.3)	0.8 (11.9)	2.1 (29.1)
12	- -	- -	- -	- -	- -
15	3.0 (28.1)	2.7 (25.3)	1.3 (11.4)	2.1 (19.5)	2.7 (25.2)
19	3.0 (28.3)	2.4 (22.7)	1.5 (13.9)	3.4 (32.5)	4.1 (38.9)
22	- -	- -	- -	- -	- -
25	1.6 (37.1)	0.5 (9.4)	0.3 (5.0)	2.2 (45.2)	2.6 (53.6)
29	2.9 (53.5)	1.5 (28.2)	0.3 (5.5)	2.3 (42.7)	2.1 (38.7)
32	1.5 (25.2)	1.0 (28.8)	0.2 (3.9)	2.5 (41.4)	3.0 (49.2)
36	1.9 (32.7)	1.6 (27.4)	0.2 (3.5)	1.5 (26.1)	2.4 (40.4)
41	2.5 (44.1)	1.2 (20.9)	0.4 (6.4)	1.9 (33.3)	3.1 (54.5)
44	2.2 (36.4)	1.1 (18.2)	0.4 (6.5)	2.7 (45.1)	3.4 (56.0)
47	3.2 (41.5)	2.7 (35.6)	0.5 (6.7)	2.2 (29.3)	2.6 (34.2)
50	2.4 (33.3)	1.9 (26.6)	0.5 (7.5)	3.4 (47.4)	3.6 (50.0)
54	3.0 (37.7)	1.7 (20.9)	0.4 (5.3)	2.5 (31.1)	5.0 (63.0)
57	2.1 (29.5)	1.6 (22.6)	0.2 (3.1)	2.2 (29.0)	2.7 (37.3)
61	2.2 (25.0)	0.5 (18.2)	0.3 (3.4)	6.0 (68.6)	6.1 (69.5)
Trypanocidal drug therapy					
64	0.7 (9.9)	0.5 (7.3)	0.1 (1.7)	3.5 (49.7)	4.7 (70.8)
69	1.3 (33.1)	1.0 (24.3)	0.6 (11.3)	2.1 (40.0)	2.0 (37.8)
72	2.3 (37.2)	1.5 (21.0)	0.5 (7.6)	2.3 (37.3)	2.7 (43.0)
76	3.2 (29.1)	1.5 (11.8)	0.6 (5.2)	4.3 (39.2)	6.6 (61.1)
79	5.5 (45.7)	2.8 (21.5)	1.2 (9.4)	5.4 (41.0)	4.8 (36.7)

FIGURE P 5.3 Peripheral blood leucocyte subpopulation dynamics, parasitaemia and changes in PCV in sheep 105 infected with *T. congolense* TREU 1457



P5.3

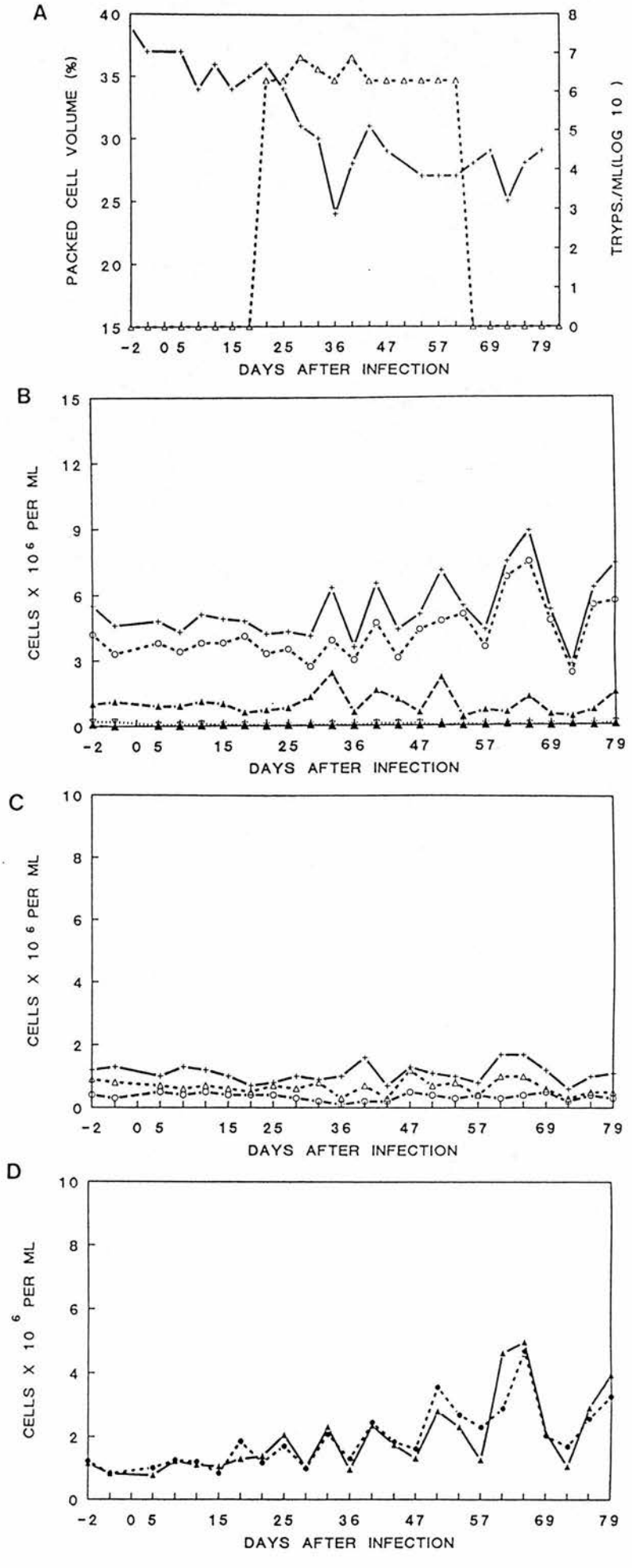

A
PACKED CELL VOLUME (%)
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
B
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
C
CELLS X 10 6 PER ML
DAYS AFTER INFECTION
D
CELLS X 10 6 PER ML
DAYS AFTER INFECTION

TABLE P 5.7 Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 105 infected with *T. congolense* TREU 1457

Days after infection	PCV (%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia tryps/ml (log_{10})
-2	39.0	5.5	4.2	-
-1	37.0	4.6	3.3	-
5	37.0	4.8	3.9	-
8	34.0	4.3	3.4	-
12	36.0	5.1	3.8	-
15	34.0	4.9	3.8	-
19	35.0	4.8	4.1	-
22	36.0	4.2	3.3	6.3
25	34.0	4.3	3.5	6.3
29	31.0	4.1	2.7	6.9
32	30.0	6.3	3.9	6.6
36	24.0	3.6	3.0	6.3
41	28.0	6.5	4.7	6.9
44	31.0	4.4	3.1	6.3
47	29.0	5.1	4.4	6.3
50	-	7.1	4.8	6.3
54	27.0	5.5	5.1	6.3
57	27.0	4.4	3.6	6.3
61	27.0	7.5	6.8	6.3
Trypanocidal drug therapy				
64	28.0	8.9	7.5	
69	29.0	5.3	4.8	
72	25.0	2.8	5.5	
76	28.0	6.3	5.7	
79	29.0	7.4	5.7	

TABLE P 5.8 Peripheral blood leucocyte subpopulation dynamics in sheep 105 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-2	1.2 (29.0)	0.9 (21.0)	0.4 (10.0)	1.1 (27.0)	1.2 (29.0)
-1	1.3 (40.7)	0.8 (25.0)	0.3 (8.2)	0.8 (24.8)	0.8 (25.2)
5	1.0 (26.3)	0.7 (16.8)	0.5 (12.4)	0.8 (20.0)	1.0 (25.4)
8	1.3 (38.2)	0.6 (19.0)	0.4 (19.4)	1.2 (36.1)	1.3 (37.0)
12	1.2 (30.5)	0.7 (18.3)	0.5 (12.0)	1.1 (28.7)	1.2 (31.5)
15	1.0 (27.6)	0.6 (15.3)	0.4 (10.6)	1.05 (26.1)	0.8 (21.9)
19	0.7 (16.7)	0.5 (11.8)	0.4 (9.4)	1.3 (31.3)	1.9 (44.8)
22	- -	- -	- -	- -	- -
25	0.8 (22.6)	0.7 (19.9)	0.4 (12.4)	2.1 (58.8)	1.7 (48.2)
29	1.0 (36.1)	0.6 (20.7)	0.3 (11.5)	1.0 (37.3)	1.0 (35.9)
32	0.9 (23.5)	0.8 (21.9)	0.2 (4.5)	2.3 (59.7)	2.1 (53.5)
36	1.0 (32.1)	0.3 (11.7)	0.1 (3.7)	1.0 (32.2)	1.3 (44.1)
41	1.6 (32.7)	0.7 (13.9)	0.2 (4.2)	2.4 (49.5)	2.5 (51.7)
44	0.7 (22.1)	0.3 (9.5)	0.2 (5.1)	1.7 (56.5)	1.8 (59.9)
47	1.3 (29.3)	1.2 (28.1)	0.5 (10.9)	1.3 (29.6)	1.6 (36.6)
50	1.1 (22.8)	0.7 (15.4)	0.4 (7.2)	2.8 (58.3)	3.6 (73.9)
54	1.0 (18.9)	0.8 (15.5)	0.3 (5.0)	2.3 (45.6)	2.7 (53.4)
57	0.8 (22.8)	0.4 (10.5)	0.4 (6.3)	1.3 (34.6)	2.3 (63.7)
61	1.7 (25.7)	1.0 (15.4)	0.3 (4.8)	4.6 (68.4)	2.9 (42.7)
Trypanocidal drug therapy					
64	1.7 (22.3)	1.0 (13.3)	0.4 (5.1)	5.0 (66.2)	4.7 (62.5)
69	1.2 (24.6)	0.6 (12.1)	0.5 (10.7)	2.1 (44.4)	2.0 (42.5)
72	0.6 (25.7)	0.3 (19.3)	0.2 (6.6)	1.1 (43.2)	1.7 (69.2)
76	1.0 (18.7)	0.5 (8.6)	0.4 (7.5)	2.9 (53.1)	2.6 (46.8)
79	1.1 (18.7)	0.5 (9.0)	0.3 (5.4)	4.0 (68.9)	3.3 (57.1)

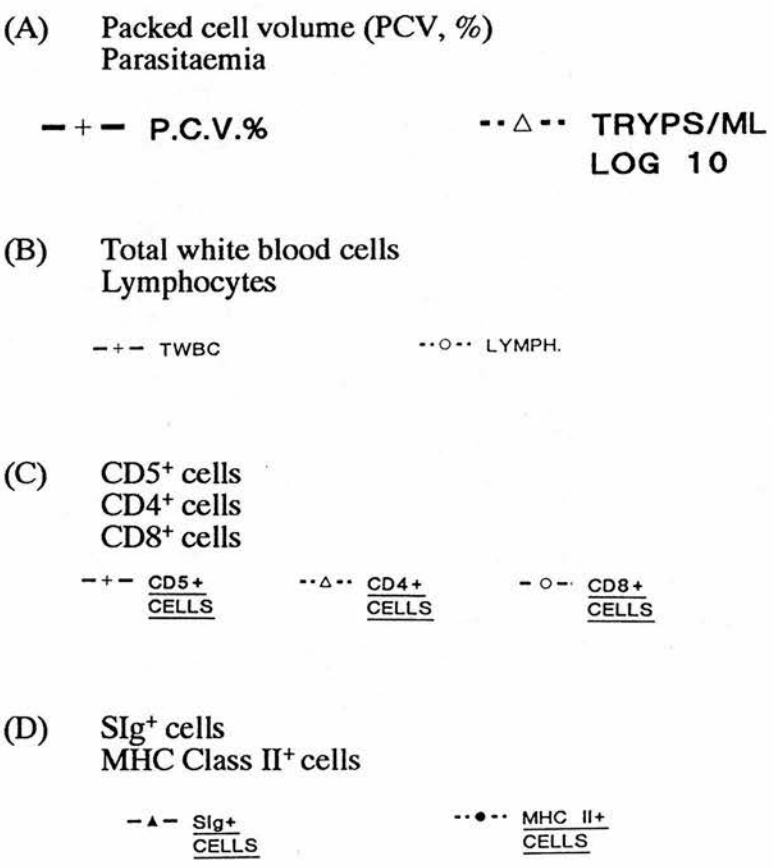
TABLE P 5.9 Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 940 infected with *T. congolense* TREU 1457

Days after infection	PCV (%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia (log_{10} tryps/ml)
-4	36.0	17.9	10.2	-
-1	36.0	18.0	11.1	-
3	31.0	15.2	9.3	-
6	33.0	9.8	8.0	-
10	35.0	15.3	10.7	-
13	32.0	13.7	10.4	-
17	33.0	11.3	9.1	-
20	32.0	15.5	10.9	-
24	33.0	15.4	12.2	-
27	31.0	17.2	12.5	2
31	31.0	13.6	10.3	2
34	35.0	12.3	10.2	5.7
38	30.0	11.2	9.9	6
39	Trypanocidal drug therapy			
55	32.0	14.1	10.2	-
69	32.0	13.4	9.5	-

TABLE P 5.10 Peripheral blood leucocyte subpopulation dynamics in sheep 940 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infections	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-4	5.5 (53.4)	1.3 (12.5)	0.7 (7.0)	4.8 (47.0)	4.8 (46.8)
-1	4.6 (41.9)	1.7 (15.4)	0.9 (20.4)	6.1 (55.3)	6.2 (55.6)
3	4.0 (42.8)	1.9 (20.6)	0.3 (2.8)	4.5 (48.0)	4.1 (43.9)
6	- -	- -	- -	- -	- -
10	3.7 (35.4)	1.4 (13.3)	0.6 (5.9)	4.2 (38.9)	4.9 (43.9)
13	- -	- -	- -	- -	- -
17	4.2 (45.9)	2.2 (23.9)	0.5 (5.4)	4.9 (53.8)	5.2 (56.8)
20	4.0 (37.7)	1.8 (16.9)	1.2 (0.8)	5.2 (47.6)	5.4 (49.6)
24	6.3 (51.5)	2.3 (19.0)	1.8 (14.4)	6.1 (50.4)	4.2 (34.5)
27	4.0 (32.3)	1.9 (15.1)	1.2 (9.5)	9.0 (72.3)	5.3 (42.2)
31	2.9 (18.9)	1.5 (14.7)	1.8 (6.8)	6.5 (62.7)	7.1 (68.9)
34	3.5 (34.2)	1.3 (12.7)	1.2 (7.9)	6.0 (59.2)	4.6 (44.7)
38	2.5 (14.8)	1.3 (13.0)	0.7 (13.1)	5.9 (59.3)	5.4 (54.4)
39	Trypanocidal drug therapy				
55	3.0 (29.4)	2.0 (19.9)	0.9 (9.0)	7.6 (74.1)	7.9 (77.9)
69	3.7 (38.5)	1.3 (14.2)	1.3 (13.4)	5.6 (58.6)	5.5 (58.4)

FIGURE P 5.4 Peripheral blood leucocyte subpopulation dynamics, parasitaemia and changes in PCV in sheep 941 infected with *T. congolense* TREU 1457



P5.4

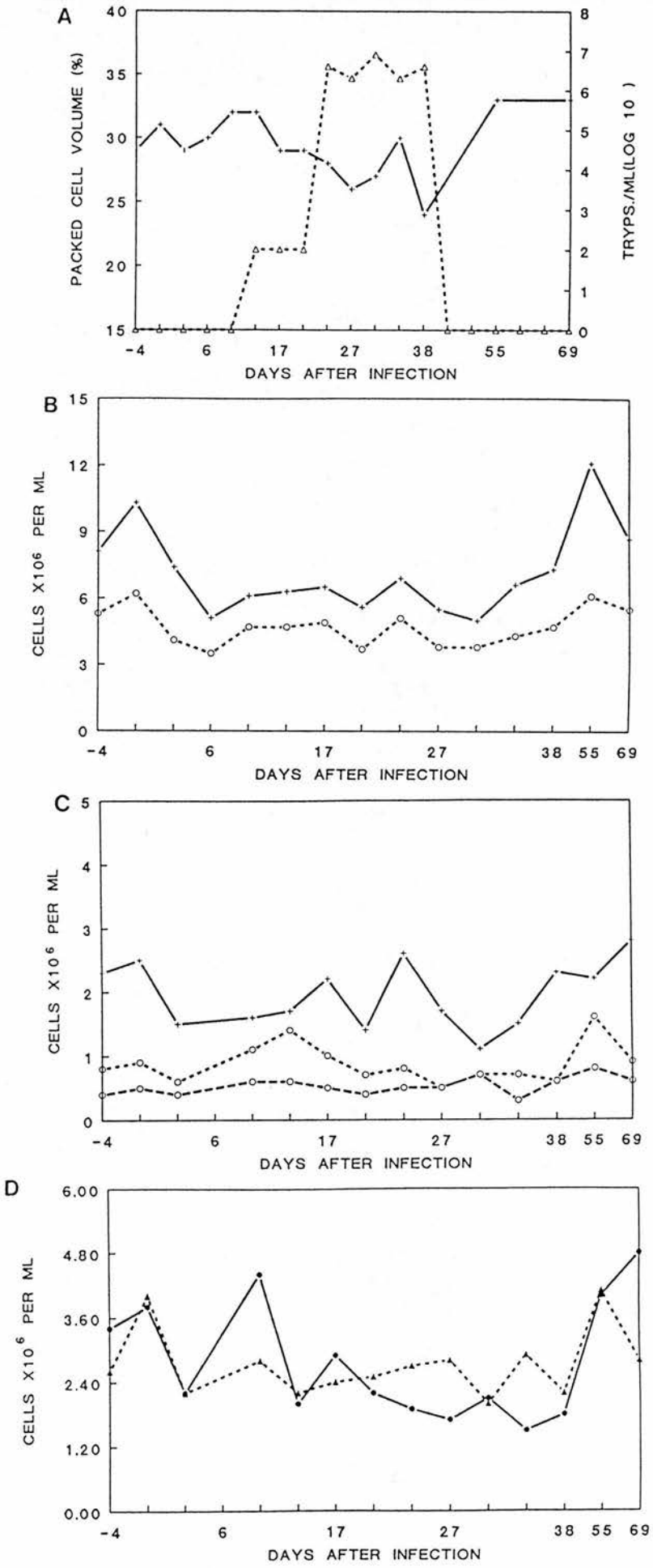

A
PACKED CELL VOLUME (%)
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
B
CELLS X10⁶ PER ML
DAYS AFTER INFECTION
C
CELLS X10⁶ PER ML
DAYS AFTER INFECTION
D
CELLS X10⁶ PER ML
DAYS AFTER INFECTION

TABLE P 5.11 Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 941 infected with *T. congolense* TREU 1457

Days after infection	PCV (%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia (log_{10} tryps/ml)
-4	29.0	8.1	5.3	-
-1	31.0	10.3	6.2	-
3	29.0	7.4	4.1	-
6	30.0	5.1	3.5	-
10	32.0	6.1	4.7	-
13	32.0	6.3	4.7	2
17	29.0	6.5	4.9	2
20	29.0	5.6	3.7	2
24	28.0	6.9	5.1	6.6
27	26.0	5.5	3.8	6.3
31	27.0	5.0	3.8	6.9
34	30.0	6.6	4.3	6.3
38	24.0	7.3	4.7	6.6
39	Trypanocidal drug therapy			
55	33.0	12.1	6.1	-
69	33.0	8.7	5.5	-

TABLE P 5.12 Peripheral blood leucocyte subpopulation dynamics in sheep 941 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	CD5$^+$	CD4$^+$	CD8$^+$	SIg$^+$	MHC Class II$^+$
-4	2.3 (44.1)	0.8 (15.2)	0.4 (6.9)	2.6 (49.0)	3.4 (64.9)
-1	2.5 (40.3)	0.9 (15.7)	0.5 (8.2)	4.0 (65.0)	3.8 (61.7)
3	1.5 (36.9)	0.6 (15.4)	0.4 (9.2)	2.2 (53.2)	2.2 (53.1)
6	- -	- -	- -	- -	- -
10	1.6 (34.5)	1.1 (24.3)	0.6 (12.6)	2.8 (59.1)	4.4 (38.9)
13	1.7 (35.6)	1.4 (29.8)	0.6 (12.2)	2.2 (47.4)	2.0 (42.4)
17	2.2 (48.4)	1.0 (20.4)	0.5 (9.9)	2.4 (48.8)	2.9 (59.5)
20	1.4 (38.2)	0.7 (17.9)	0.4 (9.9)	2.5 (67.5)	2.2 (59.0)
24	2.6 (50.8)	0.8 (17.4)	0.5 (10.2)	2.7 (53.4)	1.9 (37.2)
27	1.7 (43.5)	0.5 (13.8)	0.5 (11.9)	2.8 (74.7)	1.7 (45.2)
31	1.1 (28.5)	0.7 (18.4)	0.7 (18.8)	2.0 (53.6)	2.1 (54.5)
34	1.5 (35.9)	0.7 (16.0)	0.3 (6.8)	2.9 (67.0)	1.5 (34.5)
38	2.3 (50.1)	0.6 (13.0)	0.6 (12.9)	2.2 (46.9)	1.8 (39.1)
39	Trypanocidal drug therapy				
55	2.2 (35.3)	1.6 (26.7)	0.8 (13.2)	4.1 (67.5)	4.0 (65.2)
69	2.8 (50.9)	0.9 (17.6)	0.6 (11.4)	2.8 (50.9)	4.8 (88.3)

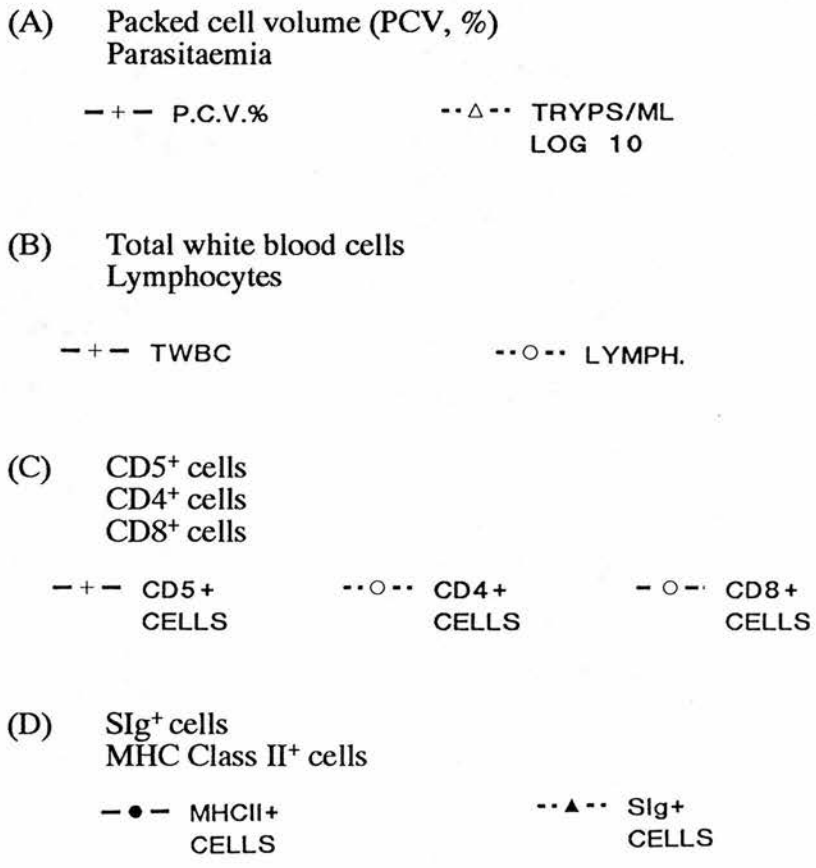
TABLE P 5. 13 Changes in packed cell volume (PCV), total white blood cell counts TWBC, lymphocyte counts (lymph) and parasitaemia in sheep 942 infected with *T. congolense* TREU 1457

Days after infection	PCV (%)	TWBC ($x10^6$/ml)	Lymph. ($x10^6$/ml)	Parasitaemia (log_{10} tryps/ml)
-4	27.0	9.1	5.6	-
-1	28.0	8.9	6.0	-
3	28.0	9.1	5.7	-
6	26.0	8.0	5.7	-
10	29.0	8.1	6.4	-
13	28.0	8.9	6.6	-
17	28.0	7.3	5.2	-
20	27.0	8.2	5.5	-
24	27.0	8.5	6.2	-
27	27.0	8.6	5.5	-
31	26.0	7.6	5.7	-
34	28.0	6.8	4.2	-
38	28.0	7.8	4.5	-
39	Trypanocidal drug therapy			
55	31.0	8.1	5.1	-
69	30.0	6.9	4.5	-

TABLE P 5.14 Peripheral blood leucocyte subpopulation dynamics in sheep 942 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-4	2.0 (35.7)	1.2 (21.0)	0.6 (10.4)	2.8 (50.1)	3.6 (64.8)
-1	3.2 (53.0)	1.1 (17.6)	0.9 (15.3)	3.0 (49.2)	3.0 (49.9)
3	2.9 (51.6)	0.9 (16.9)	0.60 (10.5)	2.8 (49.0)	2.5 (43.5)
6	- -	- -	- -	- -	- -
10	2.9 (45.5)	1.9 (30.6)	1.1 (17.9)	2.2 (35.0)	2.4 (37.2)
13	2.8 (42.5)	2.5 (37.4)	1.6 (24.3)	2.9 (43.6)	3.2 (48.2)
17	3.4 (64.6)	1.1 (20.9)	0.6 (12.1)	2.1 (40.2)	2.8 (53.2)
20	2.4 (43.7)	1.1 (20.8)	0.7 (13.1)	2.7 (49.2)	2.3 (42.6)
24	4.1 (66.4)	1.3 (21.3)	1.2 (20.4)	2.3 (37.3)	1.9 (31.3)
27	2.9 (53.2)	1.1 (19.8)	1.1 (20.1)	3.1 (57.0)	3.1 (55.5)
31	1.9 (32.6)	1.0 (17.1)	0.7 (12.0)	4.1 (72.0)	4.1 (71.3)
34	2.7 (63.5)	0.9 (21.0)	0.9 (22.6)	1.7 (40.1)	1.7 (41.5)
38	1.6 (35.6)	0.6 (13.8)	0.9 (19.0)	2.1 (46.1)	2.2 (49.1)
39	Trypanocidal drug therapy				
55	3.0 (59.4)	1.3 (25.2)	0.7 (14.1)	2.8 (54.6)	2.3 (44.4)
69	2.3 (51.6)	1.9 (41.9)	0.8 (18.2)	2.2 (49.5)	2.3 (50.1)

FIGURE P 5.5 Peripheral blood leucocyte subpopulation dynamics, parasitaemia and changes in PCV in sheep 943 infected with *T. congolense* TREU 1457



P5.5

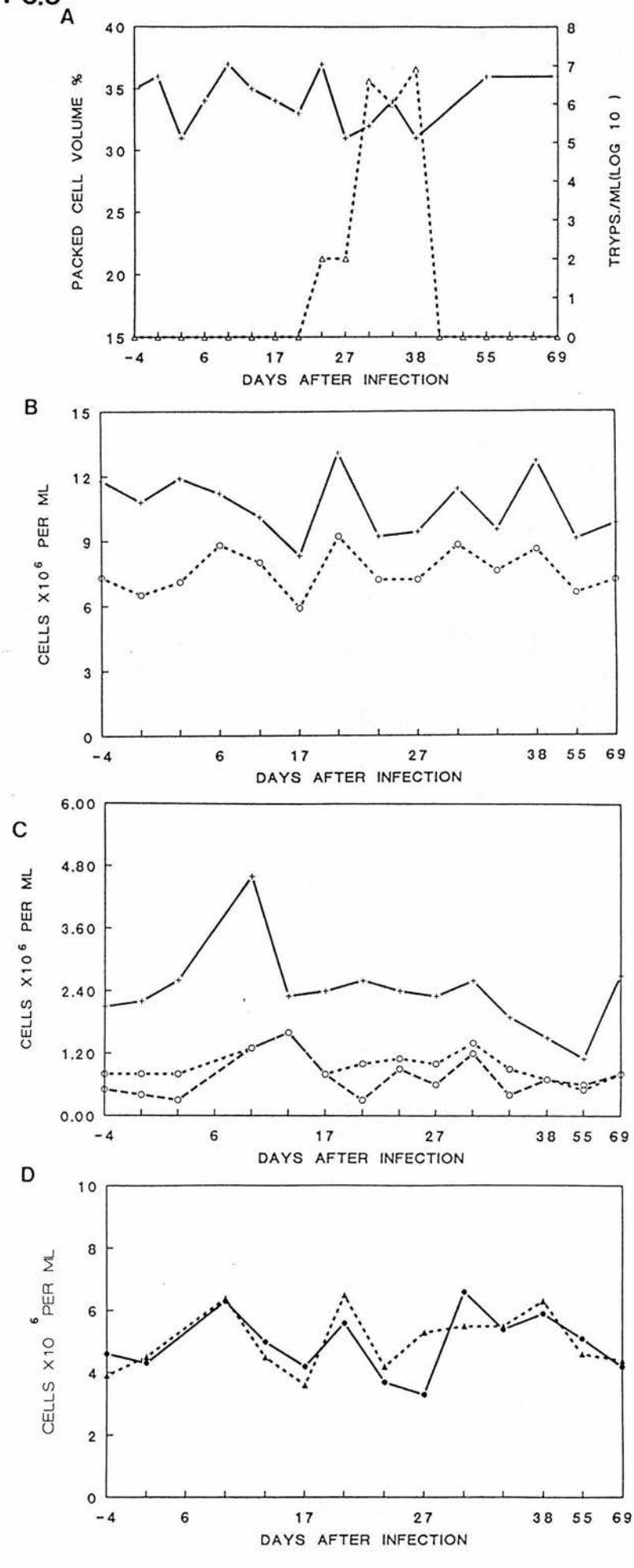

A
PACKED CELL VOLUME %
TRYPS./ML(LOG 10)
DAYS AFTER INFECTION
B
CELLS X10⁶ PER ML
DAYS AFTER INFECTION
C
CELLS X10⁶ PER ML
DAYS AFTER INFECTION
D
CELLS X10⁶ PER ML
DAYS AFTER INFECTION

TABLE P 5.15 Changes in packed cell volume (PCV), total white blood cell counts (TWBC), lymphocyte counts (lymph) and parasitaemia in sheep 943 infected with *T. congolense* TREU 1457

Days after infection	PCV (%)	TWBC (10^6/ml)	Lymph. (x10^6/ml)	Parasitaemia $\log_{10}$ tryps/ml)
-4	35.0	11.8	7.3	-
-1	36.0	10.8	6.5	-
3	31.0	11.9	7.1	-
6	34.0	11.2	8.8	-
10	37.0	19.1	13.7	-
13	35.0	10.1	8.0	-
17	34.0	8.3	5.9	-
20	33.0	13.1	9.2	2
24	37.0	9.2	7.2	2
27	31.0	9.4	7.2	6.3
31	32.0	11.4	8.8	6.6
34	34.0	9.5	7.6	6.0
38	31.0	12.7	8.6	6.9
39	Trypanocidal drug therapy			
55	36.0	9.1	6.6	-
69	36.0	9.8	7.2	-

TABLE P 5.16 Peripheral blood leucocyte subpopulation dynamics in sheep 943 infected with *T. congolense* TREU 1457. Absolute numbers x10^6/ml (and proportions, %)

Days after infection	$CD5^+$	$CD4^+$	$CD8^+$	SIg^+	MHC Class II^+
-4	2.1 (29.4)	0.8 (10.3)	0.5 (7.2)	4.6 (63.1)	3.9 (53.4)
-1	2.2 (35.5)	0.8 (11.6)	0.3 (6.8)	4.3 (66.9)	4.2 (64.0)
3	2.6 (37.1)	0.8 (11.3)	0.3 (4.3)	4.3 (60.6)	4.5 (63.4)
6	- -	- -	- -	- -	- -
10	4.6 (34.2)	1.3 (9.4)	1.3 (3.1)	6.3 (45.9)	6.4 (46.7)
13	2.3 (28.5)	1.6 (20.4)	1.6 (9.9)	5.0 (62.5)	4.5 (56.3)
17	2.4 (39.9)	0.8 (13.9)	0.8 (4.3)	4.2 (71.2)	3.6 (61.0)
20	2.6 (27.9)	1.0 (10.7)	0.3 (10.2)	5.6 (60.9)	6.5 (70.7)
24	2.4 (33.9)	1.1 (15.4)	0.9 (7.9)	3.7 (51.4)	4.2 (58.3)
27	2.3 (31.3)	1.0 (14.0)	0.6 (7.2)	3.3 (45.8)	5.3 (73.6)
31	2.6 (29.4)	1.4 (15.9)	1.2 (13.7)	6.6 (75.0)	5.5 (62.5)
34	1.9 (24.5)	0.9 (12.4)	0.4 (4.7)	5.4 (71.1)	5.5 (72.4)
38	1.5 (17.4)	0.7 (8.1)	0.7 (8.1)	5.9 (68.6)	6.3 (73.3)
39	Trypanocidal drug therapy				
55	1.1 (16.8)	0.5 (8.2)	0.6 (9.8)	5.1 (77.3)	4.6 (69.7)
69	2.7 (36.6)	0.8 (11.6)	0.8 (11.6)	4.2 (58.3)	4.4 (61.1)

APPENDIX VI
ADDRESSES OF MANUFACTURERS

1. BDH Ltd.,
Broom Road,
Poole,
DORSET, BH12 4NN.

2. Becton-Dickinson,
Between-Towns Road,
Cowley,
OXFORD, OX4 3LY.

3. C.A. Hendley (Essex) Ltd.,
Oakwood Hill Industrial Estate,
Loughton,
ESSEX.

4. Miles Laboratories Ltd.,
Stoke Court,
Stoke Poges,
SLOUGH, SL2 4LY.

5. MSE,
Manor Park,
Crawley,
WEST SUSSEX, RH10 2QQ.

6. Nordic Immunologicals,
P.O. Box 544,
Maidenhead,
BERKS, SC6 2PW.

7. Nycomed UK (Ltd),
Nycomed House,
2111 Coventry Road,
Sheldon,
BIRMINGHAM, B26 3EA.

8. Scottish Antibody Production Unit (SAPU),
Law Hospital,
Carluke,
LANARKSHIRE.

9. Serotec,
22 Bankside Station Approach,
Kidlington,
OXFORD, OX5 1JE.

10. Shandon Southern Products,
Chadwick Road,
Astmoor,
Runcorn,
CHESHIRE, WA7 1PR.

11. Sigma Chemical Co.,
Fancy Road,
Poole,
DORSET,
BH17 7NH.

12. Sterilin Ltd.,
Lampton House,
Lampton Road,
Hounslow,
MIDDLESEX, TW3 4EE.

13. TAAB Laboratories Equipment Ltd.,
40 Grovelands Road,
Reading,
BERKSHIRE.

PUBLICATIONS

Some of the work in this thesis has been accepted for publication and presented in scientific meetings as follows:

1. Mwangi, D.M., Hopkins, J. and Luckins, A.G. (1989). Cellular phenotypes in *Trypanosoma congolense* infected sheep: Studies on local skin reactions and draining lymph nodes. Paper 620. 20th Meeting of the International Scientific Council for Trypanosomiasis Research and Control (ISTRC) Mombasa, Kenya, 10th - 14th April, 1989.

2. Mwangi, D.M., Hopkins, J. and Luckins, A.G. (1990). Cellular phenotypes in *Trypanosoma congolense* infected sheep: The local skin reaction. *Parasite Immunology*, **12**, 647-658.

3. Mwangi, D.M., Hopkins, J., Luckins, A.G. and Rae, P. (1990). Preliminary studies on lymphocyte subpopulations in *Trypanosma congolense* infected sheep. Royal Society of Tropical Medicine and Hygiene, Scottish Branch, Laboratory Meeting, Edinburgh, 24th May, 1989. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 171-172.

4. Mwangi, D.M., Hopkins, J. and Luckins, A.G. (1990). *Trypanosoma congolense* infection in sheep: Changes in lymphocyte kinetics and cellular phenotypes in lymph draining local skin reactions. Royal Society of Tropical Medicine and Hygiene, Scottish Branch, Laboratory Meeting Edinburgh, 8th May, 1990. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 895.

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Cellular phenotypes in *Trypanosoma congolense* infected sheep: The local skin reaction

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Summary Mononuclear cell subpopulations in local skin reactions (chancres) in sheep infected with metacyclic forms of *Trypanosoma congolense* were studied by indirect immunoperoxidase staining using a panel of monoclonal antibodies (MoAbs) specific for ovine leucocyte subsets. Morphometric analysis revealed significant increases in numbers of cells expressing CD5, CD4, CD8, CD45R (mainly B cells), major histocompatibility complex (MHC) class II antigens, Fc receptors (FcR) on macrophages (VPM32) and FcR on B cells and macrophages (VPM33) from five days post-infection. B cells which also expressed MHC class II were found mainly in dense aggregates. The CD4/CD8 ratios were raised over pre-infection levels at 5-7 days post-infection. In sheep which had been infected, treated with trypanocidal drugs and then challenged with an heterologous serodeme of *T. congolense*, changes in cellular phenotype kinetics were similar to those seen in the skin in primary infections. Sheep superinfected with either an homologous or an heterologous, *T. congolense* serodeme showed only mild cellular infiltration and slight increases in various cellular phenotypes at the sites of inoculation.

Keywords: *Trypanosoma congolense*, metacyclics, sheep, chancres, cellular phenotypes

Introduction

The development of localized skin reactions or chancres in mammalian hosts infected with *T. congolense* following inoculation of metacyclic trypanosomes by tsetse fly bite is of interest with regard to establishment of infection and the induction of immunity (Gray & Luckins 1980, Akol & Murray 1982, Luckins and Gray 1983, Dwinger, Rudin & Murray 1988). Similar chancres to those induced by infected tsetse fly bite can be elicited by inoculation into the skin of cultured metacyclic forms of *T. congolense* (Luckins, Rae & Gray 1981, Dwinger *et al.* 1987). Chancres are characterized histologically by an initial cellular infiltrate involving neutrophils and lymphocytes, followed progressively by substantial lymphocytic infiltration as well as ingress of macrophages, lymphoblasts and plasma cells (Gray and Luckins 1980, Emery & Moloo 1981, Akol & Murray 1982).

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A number of studies of humoral antibody responses in tsetse transmitted *T. congolense* infections have shown that protective immunity operates at the level of the skin. In addition suppression of responses to challenge with heterologous serodemes is also related to chancre development (Luckins & Gray 1983, Dwinger *et al.* 1987). In order to understand fully the immune mechanisms involved in these processes, it will be necessary to obtain more detailed knowledge of the cellular responses, particularly the changes in lymphocyte subpopulations which occur in the chancre during the early stages of infection. Recently monoclonal antibodies to sheep leucocyte antigens have become available (Mackay *et al.* 1985, Mackay, Maddox & Brandon 1986, Puri, Mackay & Brandon, 1985, Mackay, Maddox & Brandon, 1987) and this paper reports on the characteristics of various cellular phenotypes in trypanosomal chancres following infection with *T. congolense*. The effects of trypanocidal drug treatment before development of macroscopically detectable chancres, challenge of these sheep and superinfection of sheep with both homologous and heterologous serodemes of *T. congolense*, on the cellular phenotype kinetics in the inoculation sites are also reported.

Materials and methods

ANIMALS

One- to two-year-old Scottish Blackface or Suffolk sheep were used in these experiments. They were housed in concrete fly-proof pens and fed on hay and concentrates with free access to water.

TRYPANOSOMES

Cultured metacyclic forms of three serodemes of *T. congolense*, TREU 1457, TREU 1881 and TREU 1885 were used to infect sheep. TREU 1457 is a derivative of stock Zaria/67/LUMP/69 (Luckins & Gray, 1983) which was originally isolated from Nigeria. Both TREU 1881 (primary isolation code DA/ZM/81/TRPZ/105) and TREU 1885 (primary isolation code DA/ZM/81/TRPZ 132) were isolated from Kakumbi, Chipata district of Zambia in 1981 (Frame, 1989).

SEPARATION OF METACYCLIC FORMS AND INFECTION

Infective metacyclic forms of *T. congolense* were separated from other insect forms in culture supernates by passing through an anion exchange column (DEAE 52, Whatman Chemical Separation Limited, Kent, England) as described by Gray *et al.* (1985). Sheep were infected by intradermal inoculation of 10^5 metacyclic trypanosomes in 0·1 ml of phosphate saline glucose, pH 8. Skin thickness at the site of inoculation was measured daily with a pair of vernier calipers.

EXPERIMENTAL DESIGN

Four sheep were infected with TREU 1885 at six sites on their flanks on days 0, 1, 3, 6, 7 and 8. Five days after the last infection, the sheep were killed to obtain chancres at various

stages of development between 5 and 13 days. Four other sheep were infected at six sites and skin biopsies from chancres which developed were removed under local anaesthesia at various stages between 12 and 30 days post-infection.

The effect of trypanocidal drug treatment on the development of chancres was followed in two sheep infected with TREU 1885 and treated with a single dose of 7 mg/kg diminazene aceturate (Berenil, Hoechst, FRG) intramuscularly five days after infection, just before the time that chancres would be detectable by inspection and palpation. Skin biopsies were taken from these sheep 12, 17, 26 and 30 days after infection. In order to examine the effect on cellular dynamics following trypanocidal drug therapy, these sheep were then challenged with the homologous (TREU 1885) and two heterologous serodemes (TREU 1457 and 1881). Skin biopsies were removed from inoculation sites on day 7 after challenge.

To determine the effect of concurrent infection on cellular changes following homologous and heterologous challenge, two other sheep were infected with TREU 1885 and 30 days later, they were superinfected with TREU 1885, TREU 1457 and TREU 1881 at three different sites. Skin samples were taken from each of these sites on day 7 post-infection. The skin samples obtained were divided into two: samples for conventional histology were placed in neutral buffered formalin or in Bouin's solution; tissues for immunohistology were snap-frozen in dry ice and isopentane, wrapped in aluminium foil, sealed in plastic bags and stored at -70°C until used. For histological examination, skin samples were embedded in paraffin wax blocks and 5 μ thick sections cut and stained with either Mayer's haematoxylin and eosin or Giemsa (BDH Chemicals Ltd., Poole, England). Control samples were also obtained from normal uninfected skin and processed as described.

MONOCLONAL ANTIBODIES (MoABs)

Mouse monoclonal antibodies to sheep lymphocyte cell surface antigens used in this study were obtained from the Sheep Biology Unit, University of Melbourne, Australia (SBU) are listed in Table 1.

Table 1. Monoclonal antibodies to sheep lymphocyte antigens

Monoclonal antibody	Antigen specificity	Human analogue	References
25·91	SBU-T1	CD5	Mackay *et al.* (1985)
44·38	SBU-T4	CD4	Mackay *et al.* (1986)
44·97	SBU-T4	CD4	Maddox *et al.* (1985)
38·65	SBU-T8	CD8	Mackay *et al.* (1986)
19·19	SBU-T19	T cells	Mackay *et al.* (1986)
20·96	LCA-p220	p220/CD45R	Mackay *et al.* (1987)
28·1	SBU-II	MHC Class II	Puri *et al.* (1985)
VPM32	Fc receptor on macrophages	FcR III	—
VPM33	Fc receptor on macrophages and B cells	FcR	—

Two other MoAbs, MoAb VPM32 which binds on Fc receptors (FcR) on sheep macrophages, and VPM33 which binds FcR on both macrophages and B cells, were obtained from the Department of Veterinary Pathology, University of Edinburgh. MoAbs were used as undiluted tissue culture supernates.

IMMUNOHISTOCHEMICAL STAINING

Antigens expressed on lymphocyte and macrophage phenotypes were localized on 5 μm thin cryostat sections using an indirect immunoperoxidase method (Barclay, 1981). After incubation with the MoAbs, and peroxidase conjugated goat anti-mouse Ig (Nordic Immunologicals, Tilburg, The Netherlands) the staining was visualized using diaminobenzidine tetrahydrochloride (BDH) as the substrate.

MORPHOMETRIC ANALYSIS

The number of $CD5^+$, $CD4^+$, $CD8^+$, SBU–$T19^+$, and $CD45R^+$ cells were determined by counting positive cells per unit area using a modification of the method used by Armstrong *et al.* (1987) and Gorell, Miller & Brandon (1988a). Cells in the diffuse infiltrate were counted at $\times 400$ magnification in at least five similar fields (each 0·08 mm^2) from at least 4 sections of each skin sample for each of the above cellular phenotypes using a graticuled eyepiece with 121 intersections (Periplan GF, Leitz Wetzler, Germany). Cells in very dense clusters were not counted. MHC class II^+ and FCR^+ cells were difficult to visualize as distinct cells in the skin and were thus not enumerated. The median values and ranges of the cell counts in the chancre and the ratios of numbers $CD4^+$ and $CD8^+$ cells were determined.

Results

CLINICAL DEVELOPMENT OF THE CHANCRE

Intradermal inoculation of metacyclic forms of *T. congolense* TREU 1885, induced local skin reactions at all inoculation sites in normal sheep. In primary infections skin thickness increased from a pre-infection value of 3 mm to a peak of 6·3 mm at day 6 post-infection and then regressed gradually to below 4 mm on day 17 post-infection. In sheep treated with Berenil 5 days after infection, skin thickness reached a peak of 5·2 mm on day 6 and decreased to below 4 mm by day 8 post-infection. When these sheep were challenged with an homologous serodeme of *T. congolense*, chancres similar to those found in primary infections developed whereas no chancres developed at sites where an homologous serodeme was inoculated. Sheep infected with *T. congolense* TREU 1885 and then superinfected with both homologous and heterologous serodemes failed to develop any macroscopically detectable chancres.

HISTOPATHOLOGY OF THE CHANCRE

The chancre was initially characterized by infiltration of numerous polymorphonuclear leucocytes (PMNs) and mononuclear cells in the papillary dermis, around the vascular trunks, reticular dermis and hypodermis (Figure 1) between 5 and 7 days post-infection

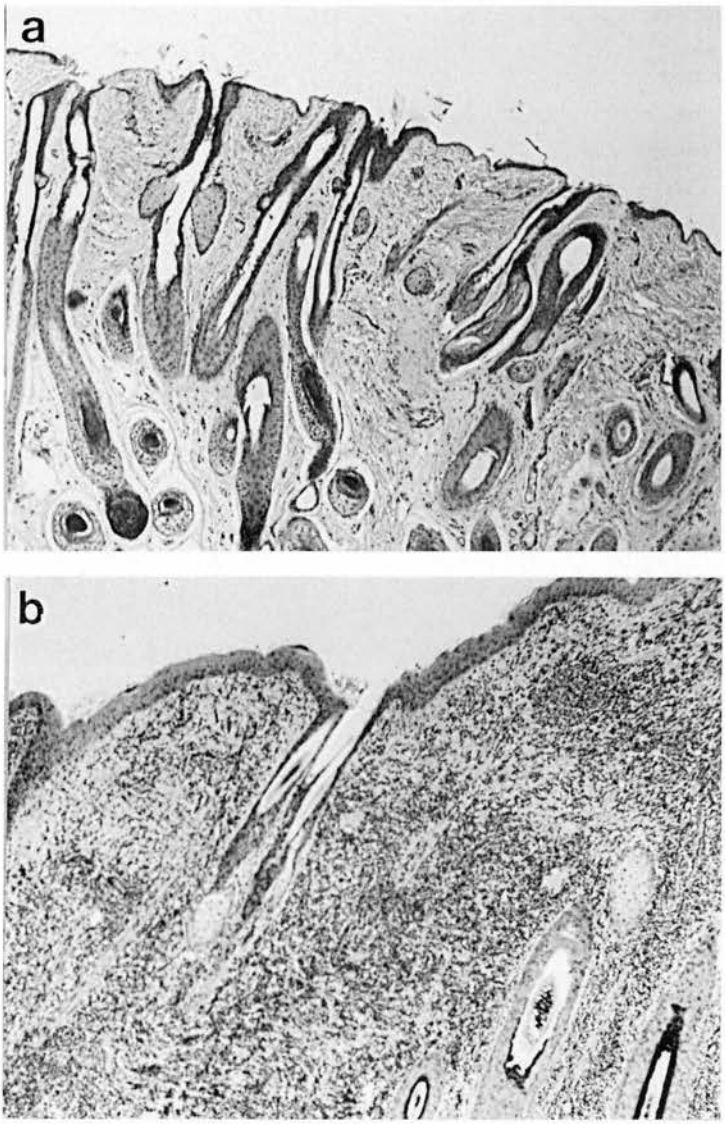

Figure 1. (a) Normal uninfected skin (H + E × 50)-(b) Severe cellular infiltration in the skin of sheep 7 days after intradermal inoculation of 10^5 metacyclic forms of *T. congolense* TREU 1885 (Giemsa, × 50.)

(p.i.). The peak of cellular infiltration which occurred 10–15 days p.i. was comprised mainly of lymphocytes, lymphoblasts, macrophages, plasma cells and very few neutrophils. From 17 days p.i. the skin reaction was no longer detectable macroscopically and the density of cellular infiltrate containing lymphoblasts, plasma cells, macrophages and fibroblast-like cells decreased progressively. The magnitude of cellular response in the skin was diminished in the sheep treated with Berenil. Cellular changes in the skin of these

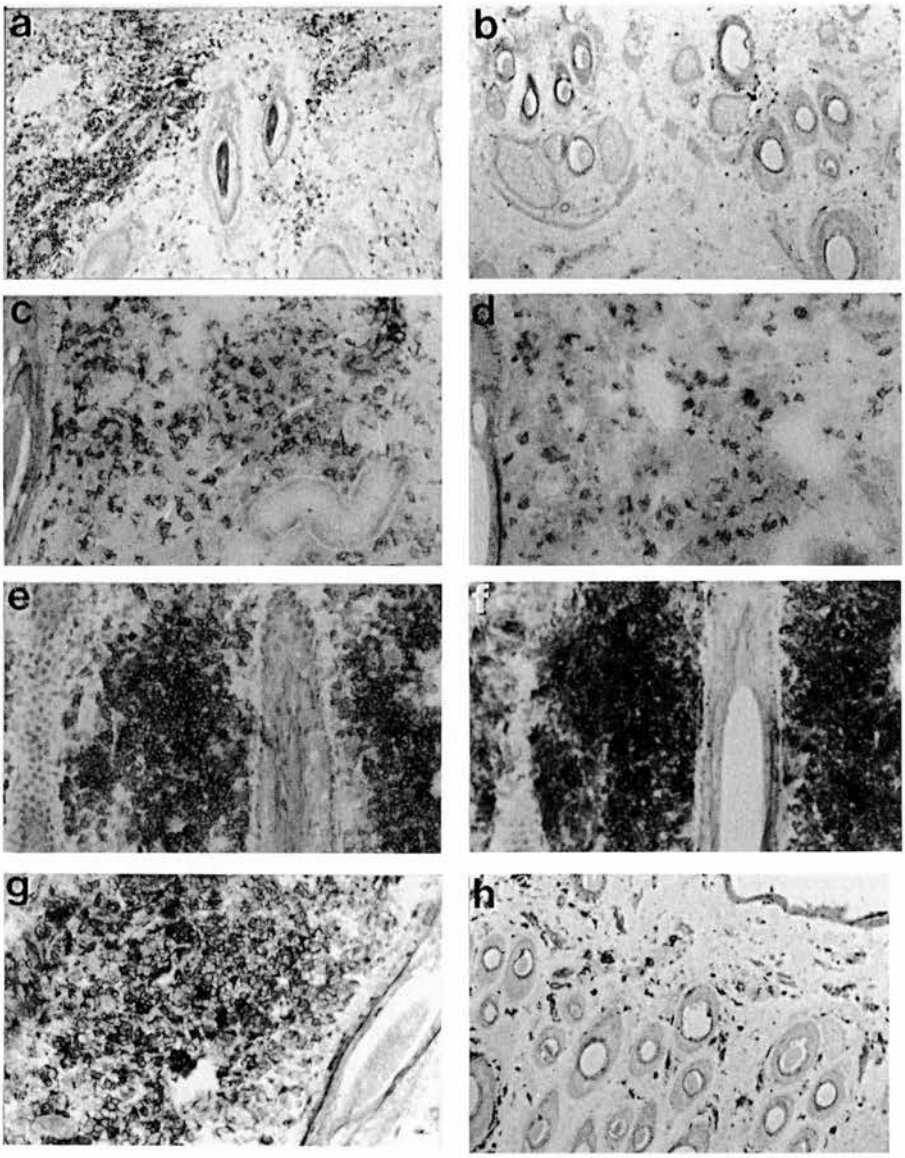

Figure 2. Cellular phenotypes in local skin reactions of sheep 7 days after *T. congolense* infection. Immunoperoxidase staining with monoclonal antibodies against surface antigens CD5 (a), CD4 (c), CD8 (d), CD45R (e), MHC Class II (f and g) and normal non-infected skin of sheep stained with CD5 (b) and MHC Class II (h). Note the dense CD45R$^+$ aggregates in the skin which also heavily express MHC Class II. (Magnification a, b, + h $\times$ 50 c, d, e, f, g, $\times$ 128.)

two sheep, at sites of challenge with heterologous serodemes, were similar to those seen in primary infections. In contrast, there was little evidence of cellular infiltration in the skin of infected sheep 7 days after they were superinfected with homologous and heterologous *T. congolense* serodemes. Similarly there was little evidence of cellular changes at inoculation sites in drug treated sheep 7 days after challenge with an homologous serodeme.

LYMPHOCYTE PHENOTYPES IN SHEEP FOLLOWING PRIMARY INFECTION

Representative illustrations of the changes in cellular phenotypes in chancres are shown in Figure 2 and quantitative morphometric analyses in Table 2. In order to simplify analysis and presentation of results, the observations on individual chancres were pooled into five separate time periods depending on the time after infection, skin thickness and histological appearance of the chancre. These periods were designated as 1 (day 0, normal skin), 2 (day 5–7, peak skin thickness with dense infiltrate of mononuclear cells and PMNs), 3 (day 10–15, regressing skin thickness but dense mononuclear cell infiltrate), 4 (day 17–23 regressed chancres, with moderate cellular infiltrate) and, 5 (day 26–30 regressed chancre with low cellular infiltrate).

Normal skin contained few lymphocytes, mainly $CD5^+$, $CD4^+$, $CD8^+$, with few $CD45R^+$ cells and macrophages expressing FcR. Following infection, there were increases in numbers of both $CD5^+$ and $CD45R^+$ cells during the chancre development (5–7 days p.i.). $CD45R^+$ lymphocytes which also expressed MHC Class II were found in aggregates. These cells in the diffuse infiltrate reached their peak numbers between days 5–7 p.i.

Within the T cell population, there were parallel increases in $CD4^+$, $CD8^+$ and SBU–$T19^+$ cells which reached their peak numbers 10–15 days after infection. The ratio of CD4/CD8 in normal uninfected skin was found to be approximately one. Five to 7 days post-infection, there were more $CD4^+$ cells than $CD8^+$ cells and the CD4/CD8 ratio increased to 2·07. The total of cells expressing $CD4^+$, $CD8^+$ and SBU–$T19^+$ was greater than that of those expressing CD5.

e 2. Median values (and ranges) of lymphocyte phenotypes in the skin reactions of sheep infected with *ngolense*

phocyte ıotype	Days after infection (periods)									
	0 (1)		5–7 (2)		10–15 (3)		17–23 (4)		26–30 (5)	
;	5*	(1–28)	103·5	(43–239)	110·5	(41–361)	70·0	(44–110)	32·0	(5–119)
+	4	(1–15)	83·0	(20–188)	86·0	(20–365)	41·5	(13–89)	30·5	(4–74)
;	6	(3–16)	45·0	(9–129)	60·5	(8–114)	59·0	(10–135)	42·0	(8–146)
-T19	2	(0–9)	6·0	(2–89)	6·0	(0–13)	4·0	(0–19)	5·0	(1–21)
5R	0	(0–6)	108·0	(32–177)	33·5	(0–155)	53·0	(34–76)	10·0	(0·69)
/CD8	1·13	(0·5–1·29)	2·07	(1·01–4·77)	1·44	(0·39–3·5)	0·74	(0·5–1·19)	0·78	(0·56–0·9)

Number of cells per 0·08 mm^2 fields (× 40 objective, × 10 eyepiece).

Table 3. Median values (and ranges) of lymphocyte phenotypes in the skin reaction of sheep infected with *T. congolense* and then treated with Berenil 5 days after infection

Lymphocyte phenotype	Days after infection			
	12	17	26	30
CD5	50·5* (14–59)	54·0 (27–88)	11·0 (10–11)	41·5 (12–60)
CD4	57·0 (8–102)	38·5 (24–50)	5·0 (3–16)	13·0 (10–39)
CD8	51·0 (10–85)	51·5 (33–68)	15·0 (7–35)	7·0 (4–21)
SBU–T19	5·0 (0–30)	15·0 (1–23)	1·0 (0–2)	6·0 (3–8)
CD45R	15·5 (4–27)	7·0 (0–13)	0 (0–1)	0 (0)
CD4/CD8	1·1	0·7	0·3	1·8

* Cells per 0·08 mm² field (× 40 objective, × 10 eyepiece).

Although MHC class II⁺, FcR⁺ cells in the chancre could not be easily quantified, there was a marked increase in expression of MHC class II (SBU-II) 5–15 days after infection. FcR was expressed on macrophages in the dermis and epidermis between 10 and 23 days p.i. Some of the positive cells in the dermis and epidermis had dendritic-like processes. Fc receptor expression on B cells was observed only in the early stages of chancre development and diminished by 15 days p.i.

CELLULAR PHENOTYPES IN SKIN REACTIONS OF INFECTED, TREATED SHEEP

The numbers of CD5⁺, CD4⁺, CD8⁺, CD45R⁺ cells in inoculation sites of sheep infected with *T. congolense* and treated on day 5 p.i. were lower when sampled from 12 days p.i.

Table 4. Median values (and ranges) of lymphocyte phenotypes in the day 7 skin reaction of sheep infected with *T. congolense* (TREU 1885) treated with trypanocidal drug and rechallenged with homologous/heterologous serodemes

Lymphocyte phenotype	*T. congolense* serodeme challenge		
	TREU 1885	TREU 1881	TREU 1457
CD5	3·5* (2–15)	96·0 (66–162)	181·0 (121–239)
CD4	4·0 (1–15)	67·5 (43–139)	109·0 (67–188)
CD8	10·0 (3–15)	76·5 (48–122)	120·0 (79–129)
SBU–T19	0 (6–1)	8·0 (2–23)	5·0 (2–14)
CD45R	0 (0–6)	64·0 (32–31)	120·5 (93–177)
CD4/CD8	0·4	0·9	0·9

* Cells per 0·08 mm² field (× 40 objective, × 10 eyepiece).

Table 5. Median values (and ranges) of lymphocyte phenotypes in the skin reactions of sheep infected with *T. congolense* TREU 1885 and superinfected with homologous/heterologous serodemes

Lymphocyte phenotype	Sheep 885			Sheep 889		
	TREU 1885	1881	1457	TREU 1885	1881	1457
CD5	8* (5–9)	50 (36–62)	52 (36–57)	4 (1–5)	2 (2–4)	25 (18–57)
CD4	5 (3–9)	42 (30–50)	34·5 (19–40)	3 (2–7)	2 (2–4)	17 (6–33)
CD8	13 (14–16)	42 (30–58)	43 (32–58)	6 (2–9)	4 (3–5)	12 (8–33)
SBU–T19	1.5 (1–2)	0	0 (6–3)	0 (0)	3·5 (2–5)	4 (3–9)
CD45R	1·5 (1–2)	1·5 (1–2)	0 (0–3)	0 (0)	1·0 (0–4)	8 (5–14)
CD4/CD8	0·4	1·0	0·8	0·5	0·5	1·4

* Cells per 0·08 mm² field (× 40 objective, × 10 eyepiece).

than those of untreated infected sheep (Table 3). All the cellular phonotypes in infected treated sheep decreased in number more rapidly compared with untreated infected sheep.

CELLULAR PHENOTYPES IN INFECTED, TREATED AND CHALLENGED SHEEP

Skin sections of infected, treated sheep, obtained 7 days after challenge with a homologous serodeme (TREU 1885) showed lower numbers of $CD5^+$, $CD4^+$, $CD8^+$ and $CD45R^+$ cells (Table 4) than in the untreated infected sheep. Sites of inoculation with heterologous *T. congolense* serodemes (TREU 1457 or 1881) which developed into characteristic local skin reactions by day 7 p.i., the numbers of $CD5^+$, $CD4^+$, $CD8^+$, $SBU–T19^+$ and $CD45R^+$ cells were similar to those in chancres from a primary infection.

CELLULAR PHENOTYPES IN *T. CONGOLENSE* INFECTED SHEEP SUPERINFECTED WITH HOMOLOGOUS AND HETEROLOGOUS SERODEMES

These sheep failed to develop macroscopically detectable local skin reactions at the inoculation sites following superinfection. Skin at sites of inoculation with the heterologous serodeme showed a higher density of $CD5^+$, $CD4^+$ and $CD8^+$ cells than skin at sites which were inoculated with the homologous serodeme, but much lower than was found in the skin of sheep following a primary infection with *T. congolense* (Table 5).

Discussion

The chancre represents a combination of an acute inflammatory response and an immunological reaction induced by local trypanosomal proliferation at infected tsetse fly bites (Emery and Moloo 1980). We have shown that T lymphocyte subpopulations

($CD5^+$, $CD4^+$, $CD8^+$) form a major component of the mononuclear cell infiltrate into the chancre. Since these cells were diffusely distributed in the dermis except around blood vessels they might have been passively recruited into the lesion. This recruitment however appeared to be selective in that another T cell subpopulation, SBU–T19 which forms about 15% of peripheral blood lymphocytes was found only in low numbers throughout the period of chancre development.

There are two possible explanations why the total percentage of $CD4^+$, $CD8^+$ and SBU–$T19^+$ cells exceeded the percentage of $CD5^+$ cells, namely, co-expression of CD4 and CD8 antigens (Hopkins and Dutia, 1989), loss of CD5 antigens (Blue *et al.* 1986), or presence of $CD8^+CD5-$ lymphocyte phenotypes (Gorrell *et al.* 1988a). High numbers of $CD45R^+$ cells were found in chancres 5–7 days after infection. The majority of these cells were B cells as observed in the pattern of staining of lymphoid follicles of prefemoral and prescapular lymph nodes (Mwangi, Hopkins and Luckins, unpublished). Since these cells were found in aggregates resembling lymphoid follicles, it is likely that period of local B cell proliferation occurs within the chancre. The observed increase in the number of MHC class II^+ cells in the chancre was probably due to the presence of a large population of infiltrating and proliferating B cells which express class II antigens (Emery *et al.* 1987, Ellis *et al.* 1987) and also an indication of activated T cell and macrophage population (Singer & Hodes 1983). The Fc receptor expression (VPM33) observed early in the chancre was probably as a result of the presence of a high number of B cells since this expression diminished rapidly with the decrease in number of these cells.

Chancre size is dependent on the number of inoculated metacyclic trypanosomes (Dwinger *et al.* 1987). Treatment of the infected sheep therefore reduced the number of viable trypanosomes at the inoculation site and hence the size of the chancre and degree of cellular infiltration. These sheep developed characteristic reactions similar to those observed in primary infections, 7 days after challenge with an heterologous *T. congolense* serodeme but not with an homologous *T. congolense* serodeme. Thus these two sheep appeared to be immune to homologous challenge, but since trypanosome infected animals treated before 15 days after infection are fully susceptible to homologous challenge (Emery *et al.* 1980b), it is possible that in our study the development of the chancre was delayed.

Active infection in sheep prevented the development of chancres following heterologous challenge. This finding concurs with studies by other workers (Luckins and Gray 1983, Dwinger *et al.* 1989) which have shown that there is interference in the establishment of super infections in infected animals.

The present experiments have demonstrated that marked changes occur in cellular phenotypes kinetics in chancres in sheep at an early stage of infection with *T. congolense*. The qualitative and quantitative differences in lymphocyte subpopulation in the skin may be involved in determining the outcome of challenge with metacyclic trypanosomes. Protection against infection appears to occur at the level of the skin and draining lymph node (Akol and Murray 1983) and the presence of aggregate of B cells as shown here, suggest that it is possible for protective humoral responses to be effected at the level of the skin. Further work is needed to examine lymphocytes in activated cell populations from afferent and efferent lymphatics draining chancres in order to determine their phenotypes and antigen specificity, the factors which such cells elaborate and the role they play in immunity and susceptibility to trypanosomiasis.

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CELLULAR PHENOTYPES IN *TRYPANOSOMA CONGOLENSE* INFECTED SHEEP: STUDIES ON THE LOCAL SKIN REACTION AND DRAINING LYMPH NODES

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Changes in lymphocyte subpopulations in the skin at the site of intradermal inoculation of cultured metacyclic trypanosomes and in the lymph nodes draining the inoculation site was monitored in sheep infected with *Trypanosoma congolense* by conventional histological and immunohistochemical techniques. A panel of monoclonal antibodies with specificities for ovine leucocyte phenotypes was used in immunoperoxidase staining of frozen tissue sections to determine the localisation of different lymphocyte subsets and their relative abundance.

In primary infections, the initial leucocyte infiltration into the dermis to form the the chancre at 5 days post-infection consisted histologically of neutrophils and lymphocytes. At the peak of the reaction, between days 7 and 10, there was a preponderance of mononuclear cell infiltration, mainly lymphocytes. T cells formed the predominant population of the lymphocytes and they were widely distributed while B cells were present mainly in discrete clusters. The proportion of T cells expressing SBU-T4, the ovine equivalent of CD4 was greater than that expressing SBU-T8, the ovine equivalent to CD8 antigens. Few macrophages were observed at the peak of the reaction, but there was marked expression of MHC Class II cells. In the regressing phase of the chancre (from day 13) there were fewer B cells, loss of Fc receptor (FcR) expression, marked expression of MHC Class II, an increased number of macrophages and the predominant phenotype of T cells was SBU-T8.

In draining lymph nodes, there were parallel changes in cellular phenotypes. Expression of MHC Class II, macrophages and B cells increased during infection with concomitant decrease in the numbers of cells expressing SBU-T1 (ovine analogy of CD5) and SBU-T8 cells and macrophages.

Sheep which had been infected, treated with trypanocidal drug and then challenged with an heterologous serodeme of *T. congolense* showed similar cellular phenotype kinetics to that seen in primary infections. In infected sheep challenged with either an heterologous or the homologous serodeme only mild cellular infiltration was observed with cells expressing MHC Class II, macrophage marker and FcR.

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Preliminary studies on lymphocyte subpopulations in *Trypanosoma congolense* infected sheep

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Sheep were experimentally infected by intradermal inoculation with metacyclic forms of *Trypanosoma congolense*. Peripheral blood and efferent lymph nodes draining those inoculation sites were analysed for sequential changes in lymphocyte subpopulations using a panel of monoclonal antibodies specific for ovine lymphocyte subsets. Our studies revealed that, in both efferent lymph and peripheral blood, there were progressive increases in percentage of cells expressing class II major histocompatibility complex (MHC) antigens and surface immunoglobulin positive (SIg^+) cells. Concurrent decreases in T cell subpopulations (SBU–T1, SBU–T4 and SBU–T8) were also observed. Changes in efferent lymph were more marked and occurred earlier (5 d after infection) than in peripheral blood (15 d after infection). The high level of class II MHC expression in infected sheep was probably due to an increase in the percentage of B cells expressing class II antigens. Decreases in the T cell subpopulation were probably relative to the increase in B cells. Alterations in lymphocyte subpopulations could play a role in the immune response and susceptibility to trypanosomiasis.

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Trypanosoma congolense infection in sheep: changes in lymphocyte kinetics and cellular phenotypes in lymph draining local skin reactions

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Localized skin reactions or chancres develop in mammalian hosts following intradermal inoculation of metacyclic forms of *Trypanosoma congolense*. Using a panel of monoclonal antibodies specific for ovine leucocyte subsets, we have examined the cellular phenotype dynamics in afferent and efferent lymph of the draining lymph nodes in sheep. Trypanosomes were detected in afferent lymph by days 4–5 and by days 5–6 in efferent lymph, with peaks on days 8 and 10 respectively. The cellular output in afferent lymph increased from day 7 and reached a maximum on days 9 and 14. These changes were due to an 8-fold increase in CD4+ cells and a 5-fold increase in output of other cellular phenotypes (CD5+, CD8+, CD1+, MHC Class II+ and Sig+ cells). In contrast, the increase in cell output in efferent lymph commenced on days 5–6 and reached a maximum on days 9 and 15, and was due to a higher output of SIg+, MHC class II+ and CD45R+ cells (20 fold) compared to the T cell subsets (4 fold increase in CD5+, CD4+ and CD8+ cells). These observations suggest that the lymph node response is due to the influx of trypanosomes rather than to migration of cells from the chancre.